Carotenoids in Relation to Mitochondria

A thesis presented by

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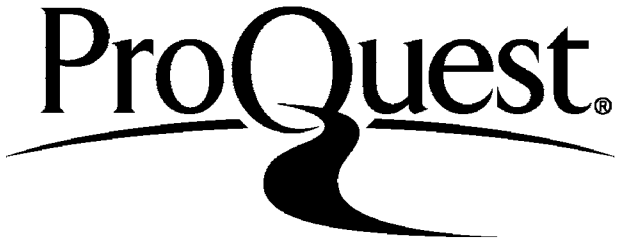

Abstract

The location of carotenoids in mitochondria is poorly documented in the literature. Therefore, initially, plant and animal tissues and a microbial culture were examined to determine whether carotenoids were situated in mitochondria and subsequently a physiological reaction of plant mitochondria, in which the participation of carotenoids may be implicated, was investigated.

To establish the presence of carotenoids in mitochondria of cauliflower buds, heart muscle, and the yeast Rhodotorula rubra the carotenoid content of arbitrary centrifugal fractions was compared with their mitochondrial enzyme activity. Cauliflower bud tissue, which contained the carotenoids β-carotene, lutein, violaxanthin, and neoxanthin, was fractionated both by differential and discontinuous sucrose density gradient centrifugation. A correlation was observed between the sedimentation patterns of carotenoid-containing particles and mitochondria, marked by succinic dehydrogenase, when lipoxidase-catalysed carotenoid oxidation and the production of a material which absorbed ultra violet light were inhibited.

Ox heart contained two types of carotenoid. One appeared to originate from the blood and could be degraded by incubation at 0° C following haemolysis. In its absence, the second type of carotenoid, composed of α- and β-carotene and a xanthophyll, was present in particles which sedimented in the same manner as the mitochondria. Carotenoid was not detected in pig heart, however.

The presence of excess extramitochondrial carotenoid in the yeast Rhodotorula rubra masked the possible presence of mitochondrial carotenoids.

Illumination was observed to decrease the rate of respiration of cauliflower bud mitochondria after protection from the light for 60 minutes. However, the period of protection was reduced by preincubation with the centrifugal supernatant, preillumination, and the action of a lipoxidase preparation on exogenous ammonium linoleate in the assay medium. Exogenous carotenoid restored protection to mitochondria from old cauliflowers and safeguarded respiration against loss of protection caused by lipoxidase activity.

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CHAPTER 1.

HISTORICAL INTRODUCTION

1. General Introduction

A definition of carotenoids accepted by the Union Internationale de Chemie states that "Carotenoids are yellow to red pigments of aliphatic or alicyclic structure, composed of isoprene units (usually 8) linked so that the two methyl groups nearest the centre of the molecule are in positions 1:6, whilst all other lateral methyl groups are in positions 1:5; the series of double bonds constitutes the chromophoric system of the carotenoids". (1)

The chemical and physical properties of carotenoids result from two characteristic features of their molecular structure. The delocalisation and mobility of the π electrons over the entire carbon chain readily permits polarisation of the molecule (2), and the energetic values of the highest occupied and the lowest empty molecular orbitals suggest that these compounds should be excellent electron donors and acceptors. (3)

In nature carotenoids have not only been found to be bound to a number of different compounds; proteins (4, 5), lipoproteins (6), and a monosaccharide (7), but also they have been isolated from several distinct types of source. The majority of these sources will therefore be reviewed, with particular attention being paid to the subcellular location of carotenoids, and suggestions concerning the metabolic role of these pigments will be discussed.

2. Carotenoids in Tissues

(a) Petals, Fruits and Roots.

Relatively high concentrations of carotenoid are found in certain flower petals, fruits and roots.

The carotenoids of flower petals, which may be attached to proteins (8), are restricted to chromoplasts. No metabolic function has been suggested for petal carotenoids. It is thought, however, that they may be an accumulated metabolic by-product, the coloured strains having proliferated because of the attraction of animals which facilitated pollination (9). A similar explanation could account for the accumulation of carotenoids in fruits.

Carotenoids are stored in certain roots such as carrot and sweet potato, but little is known of any activity in which they may participate or the reason for their accumulation.

(b) Participation in the Phototropic Effect.

Both carotenoids and riboflavin, universally present in higher plant tissues (10), have been suggested as primary light receptors in phototropic tip curvature. The action spectrum of phototropic tip curvature of Avena coleoptiles shows four maxima at 370, 425, 445, and 470mμ (10, 11). β-carotene, with maxima in petroleum ether of 426, 452, and 482 mμ has been implicated as a primary light receptor, although it shows no absorption maximum at 370mμ. In support of the role of carotenoids in phototropism, a correlation has been observed between carotenoid concentration and light sensitivity along the Avena coleoptile (11). An albino oat mutant, possessing one third of its normal carotenoid complement, showed a decrease of two thirds in the sensitivity of tip curvature (12), and a Helianthus mutant, which probably contained excessively low levels of carotenoid, showed a decreased light sensitivity (11). Alternatively, riboflavin has been nominated as the primary light receptor, having absorption maxima at 370 and 450mμ (10), as it photosensitises the in vitro oxidation of indole acetic acid. β-carotene is incapable of this, however, and protects indole acetic acid against riboflavin-catalysed photolysis (10).

A number of theories concerning the identity of the photoreceptor have been suggested to explain the action spectra.

(i) carotenoid and riboflavin, or an alternative pigment absorbing maximally at 370mμ, simultaneously absorb light (13).

(ii) light energy absorbed by carotenoids is transferred to riboflavin which is the primary photosensitiser (10).

(iii) a carotenoprotein complex exists with absorption maxima at 370, 425, 445, and 470mμ (14).

(iv) a secondary pigment, such as a riboflavin derivative, which absorbs light at 370mμ, fluoresces producing light with a wavelength between 400 and 500mμ, which is absorbed by carotenoids (10).

(v) an undiscovered compound, completely unrelated to either carotenoids or riboflavin, participates (13).

In the phototropic response of mucoraceous fungi, evidence to discriminate between carotenoids and riboflavin as the primary light receptor is similarly inconclusive. The absence of carotenoids from mutants (14) or diphenylamine-treated cultures (14) did not reduce the phototropic response. It has been emphasised, however, that although the β-carotene concentration of the diphenylamine-treated cultures was reduced there was an increase in the more saturated carotenoids (9). Conclusions from these observations are controversial because of the errors inherent in the methods available for determining action spectra (9).

c. Participation in Reproduction

"It has been noted that, among the majority of plants and animals, relatively concentrated stores of carotenoid are found in certain particular tissues, structures, and secretions associated with reproduction ..... This suggests that these pigments may play definite biochemical roles in processes associated with sexual maturation, fertilisation, and embryonic development".

Although this statement was made by Emmerson and Fox in 1940 (16), no conclusive evidence has since become available to imply that carotenoids actively participate in metabolic reactions associated with reproduction in higher plants and animals.

The association of carotenoids with the reproductive structures of a small number of fungi has lead to suggestions that they may participate in the process of sexual reproduction of these lower organisms. Oil droplets containing carotenoid form in the male gametangia of the aquatic fungus Allomyces, although the asexual structures and female gametangia are colourless (17). Similarly, parent strains of Phycomyces blakesleeanus, when grown independently, possessed β-carotene concentrations of 200 and 150 μg.lg. of tissue in the male and female cells respectively. When grown together the average mycelial concentration was 160 μg.lg. but this increased to 860 μg.lg. in the male gametangia (18). Pyronema confluens, however, requires light for the initiation of sexual reproduction but this occurred in a carotenoid-deficient mutant (17). One can therefore conclude that carotenoids are linked in some way to reproduction in certain fungi, but that this relationship is not universal.

Burnett (9) suggests that β-carotene concentration may represent the accumulation of a metabolic product resulting from the increased rate of metabolism known to occur during this phase.

d. Invertebrates

Carotenoids exist in invertebrates in both free and esterified forms. Of these the carotenoproteins have received considerable attention, but it is thought that many of their suggested functions could also apply to free carotenoids.

Carotenoprotein molecules have been isolated from the eggs, ovaries, hyperdermis, and carapace of many invertebrates. Cheesman, Lee, and Zagalsky, in a recent review (5), have suggested a variety of functions in which the carotenoid moiety may function;

(i) the stabilisation of the attached protein molecule and control of its configuration,

(ii) protective colouration both against radiation and predators,

(iii) development,

(iv) photosensitivity,

(v) electron transport,

(vi) enzymic activity.

However, the evidence for these roles is slight.

3. Carotenoids in Subcellular Particles - Chloroplasts and Chromatophores

(a) Presence in Chloroplasts.

At the subcellular level the location of carotenoids has been most thoroughly documented in the chloroplasts of green plants. All the pigments related to photosynthesis are associated with the grana, which are composed of membraneous structures. Park et al (19) have identified quantasomes, elipsoidal bodies, on the inner surfaces of the lamellae membranes of spinach chloroplasts. Each quantasome is believed to be "the morphological expression of the physiological photosynthetic unit" (19) where light reactions, electron transport, and photophosphorylations occur.

The exact carotenoid composition varies with the species and the metabolic state of the plant. However, in one analysis of the pigments of spinach chloroplast quantasomes, 230 chlorophyll molecules were shown to be present with 14 β-carotene, 22 lutein, 6 violaxanthin, and 26 neoxanthin molecules (20).

(b) Presence in Bacterial Chromatophores.

After disruption of the cell wall, particles can be obtained from photosynthetic bacteria which can perform all the

reactions of bacterial photosynthesis in the presence of added co-factors and substrates (21). These particles, which contain bacteriochlorophyll, carotenoids, and phospholipids, probably do not exist *in vivo*, but during cellular disruption may be formed from vesicular structures originating from the protoplast membrane (20).

(c) Participation in Photosynthesis.

Circumstantial evidence suggests that carotenoids may participate in photosynthesis. Photosynthetically active units of higher plants almost invariably contain carotenoids (22), they are the second major pigment of autotrophic plants (23), and in *Euglena*, for example, the rate of their formation has been shown to be parallel to that of chlorophyll (24).

Evidence of the participation of carotenoids in bacterial photosynthesis is less conclusive because the main carotenoid in *Rhodospirillum rubrum*, spirilloxanthin, is ineffective in photosynthesis (25), and carotenoidless mutants are capable of photosynthesis (26).

A number of postulated metabolic roles for carotenoids in photosynthesis are supported by inconclusive evidence. These include the transfer of absorbed light energy to chlorophyll a, the reduction of NADP, oxygen transfer and evolution, photosynthetic electron transport, and protection of cellular components against photo-oxidations mediated by chlorophyll.

(d) Transfer of Energy absorbed by Carotenoids to Chlorophyll a.

Blue light has been observed to stimulate chlorophyll a fluorescence *in vivo* (27, 28) and, in an *in vitro* system, Teale demonstrated that carotenoids facilitated this fluoresence (29). He prepared an artificial system in which chlorophyll a and carotenoids were dissolved in detergent micelles, giving localised concentrations resembling those in chloroplasts. Fluorescence resulting from illumination was not detected in simple acetone

solutions of the two pigments, but did occur in the micelles. Teale therefore suggested that energy transfer is possible when the pigments are in sufficiently close proximity.

(e) Reduction of NADP.

Examination of the action spectra of the Hill reaction in isolated chloroplasts has shown peaks in the region of the spectrum where carotenoids are known to absorb maximally (30, 31). It has therefore been suggested that carotenoids may be primary light receptors for this reaction (31).

Further evidence exists supporting the theory that light absorbed by carotenoids participates in the reduction of NADP. Kahn and Purcell (32) demonstrated an enhancement in the formation of NADPH in isolated chloroplasts caused by the addition of an uncharacterised carotenoid fraction which had been isolated in small quantities from green plant tissue. Using isolated spinach chloroplasts, Lundegardh (33) observed a number of negative bands in the difference spectrum of previously illuminated and dark chloroplasts. Amongst these was one at 500mμ which had not been reported previously, and he attributed this to carotenoids. The depression was influenced by variations in the ratio of NADP to NADPH, and Lundegardh therefore suggests that carotenoids may be involved in the reduction of NADP.

The ability of light absorbed by carotenoids to photosensitise reductions has been demonstrated by Komissarov *et al* (34). They obtained a reduction of thionine in the presence of ascorbic acid when β-carotene was absorbed on polyacrylonitrile in an illuminated artificial system.

(f) Transfer and Evolution of Oxygen.

Epoxy carotenoids are found only in organisms which evolve oxygen photosynthetically (35), and a mutant of *Scenedesmus obliquus*, incapable of evolving oxygen, was deficient in a number of cellular

constituents, including carotenoids (36). These results suggest the possibility that carotenoids may be associated with the chemical mechanism for oxygen release, and interconversions, correlated to the metabolic state of the cell, have been demonstrated between lutein or zeaxanthin and their mono- and di-epoxy derivatives (37, 38). Yamamoto *et al* (39) have shown that in lutein, violaxanthin, and neoxanthin the oxygen atoms of the -OH and epoxy groups are derived from molecular oxygen and water respectively. The origin of the epoxy oxygen was also confirmed by Saakov *et al* who showed an exchange of O^{18} between water and the epoxy oxygen during illumination of chlorella cells (40). A scheme for the biosynthetic and metabolic relationships between leaf xanthophylls has therefore been suggested (39), and a section of this, concerning the β -carotene series, is shown below.

$$\text{VIOLAXANTHIN} \underset{\text{Dark } (O)\ H_2O}{\overset{\text{Light, } N_2 \nearrow O_2}{\rightleftharpoons}} \text{ANTHERAXANTHIN} \underset{\text{Dark } (O)\ H_2O}{\overset{\text{Light, } N_2 \nearrow O_2}{\rightleftharpoons}} \text{ZEAXANTHIN}$$

Krinsky (41), using the alga *Euglena gracilis* in the stationary phase, demonstrated that only the second interconversion occurred in this organism. A "de-epoxidase" was detected requiring photosynthetically reduced co-enzymes $FMNH_2$ or NADPH (35), but the reverse reaction, epoxidation, was a non-enzymic photo-oxidation (42). Krinsky therefore suggests that this cycle offers a mechanism for the protection of the algal cells from lethal photosensitisation. The excited chlorophyll molecule would be deactivated by zeaxanthin which would be simultaneously oxidised

to the epoxide antheraxanthin. The "de-epoxidase" would then regenerate the protective substrate, zeaxanthin (42).

(g) Participation in Electron Transport.

Certain observations suggest that carotenoids may be associated with photosynthetic electron transport although they may not be components of the main pathway. Nishimira and Chance (43) observed a shift to longer wavelengths of the absorption maxima of the carotenoids of photosynthetic bacteria. This was induced by light, and changes in the carotenoid steady state were induced by heptylhydroxyquinoline-N-oxide which inhibits photosynthesis. Morita et al (44) observed a similar shift to longer wavelengths of the carotenoid absorption maxima when cytochrome oxidation resulted from illumination of a photosynthetic bacterium with red light. These shifts may reflect changes caused by participation of carotenoids in electron transport of photosynthesis, or may be caused by changes in the surrounding environment during electron transport. If carotenoids do participate in electron transport, their role may not be obligatory as carotenoidless mutants of Rhodopseudomonas spheroides and Rhodospirillum rubrum functioned normally (43).

A hypothetical mechanism for carotenoid participation in the transfer of light energy absorbed by chlorophyll has been suggested by Platt (23). The direct transfer of absorbed energy from chlorophyll to carotenoid is energetically impossible. He suggests therefore a mechanism whereby an electron donor, D, and an electron acceptor, A, approach the carotenoid molecule simultaneously, resulting in the formation of a trimolecular charge transfer complex. The stability of this structure would increase the wavelength of the carotenoid absorption maxima and reduce the energy of the excited triplet state below that of chlorophyll. The complex would hence function as an energy sink and, if the transfer energy was adequate, the resulting ions D^+ and A^- would separate

without radiation leaving the polarised carotenoid molecule to return to the ground state, having mediated electron transport.

(h) Protection against Photo-oxidation.

Photokilling in the absence of carotenoids in photosynthetic bacteria was first reported in 1955 (45). A blue-green mutant of R. spheroides ceased to grow during aerobic illumination and its bacteriochlorophyll content was bleached. Simultaneously, the wild type continued to grow, although bacteriochlorophyll and carotenoid synthesis were arrested until anaerobic conditions were restored. Exclusion of light below a wavelength of 800mμ did not alter the effect, and therefore bacteriochlorophyll was implicated as the photosensitising light receptor. Later, it was shown that diphenylamine, which inhibits carotenoid synthesis, produced light sensitivity in Chromatium (46). Protection by carotenoids against photo-oxidation was also observed in Teale's artificial system discussed above (29), the rate of chlorophyll photo-oxidation being reduced when carotenoids were incorporated in the micelle.

Claes (47) has observed that 7 or more conjugated double bonds in the carotenoid molecule are essential for protection in an anaerobic atmosphere, and 9 in an aerobic environment. For example, phytofluene, with 5 conjugated double bonds, offered no protection to chlorophyll in an in vitro system, even at high concentrations.

Sensitivity to light in the absence of carotenoids was found in higher plants in a variegated Oenothera (48), a white corn mutant (49), and a white mutant of Helianthus annus (50). In the absence of chlorophyll, this suggests that some other cell constituent was the photosensitising light receptor.

Fujimori and Livingstone (37) state that the formation of the excited triplet state is an essential intermediate step in the in vitro reactions sensitised by chlorophyll. Assuming that

this represents an *in vivo* requirement in higher plants, Claes (47) suggests that oxygen, carotenoids, and the primary metabolic energy acceptor are each efficient in quenching this state. In the absence of competition from carotenoids, oxygen excitation becomes more probable.

Evidence exists suggesting that quenching of the chlorophyll a triplet state may result in the formation of a metastable state of β-carotene (43). Alternatively, Platt's theory, discussed above, would provide a mechanism whereby the chlorophyll triplet state would transfer energy to a carotenoid. This theory supports Calvin's suggestion that the protective buffering against a photo-oxidant may be a secondary effect resulting from the primary function of removing the "excitation energy from chlorophyll in some useful way prior to its use in activation of some oxidation reaction by molecular oxygen" (45).

4. Carotenoids in Subcellular Particles - Non-photosynthetic Bacteria and Fungi

(a) Subcellular Location.

While carotenoids have been observed in many non-photosynthetic bacteria they are absent from others. Gilby, Few, and McQuillen (52) examined the bacterial protoplast membrane of *Micrococcus lysodeikticus*, produced by lysozyme treatment. Extraction of the deposited membranes produced a solution in light petroleum with absorption maxima at 416, 440, and 471mμ resembling those of a lutein solution.

The following year Mathews and Sistrom ground cells of *Sarcina* lutea with alumina and obtained carotenoprotein particles. Sonic disintegration of the cells produced these lysozyme-insensitive particles at a rate which was proportional to the rate of decrease of the optical density (53). Their experiments suggested that the particles did not pre-exist, neither did they originate from the cell wall, but were formed from the protoplast membrane. More

recently, sonic disintegration of a pleuropneumonia-like organism also demonstrated that carotenoid was associated with the protoplast membrane (54).

(b) Protection against Photosensitisation.

Carotenoids may also protect non-photosynthetic bacteria against photosensitisation. In the absence of chlorophyll, however, it is thought that other pigments, such as porphyrin derivatives, may be excited by light.

The bacteriøcidal effect of the blue region of the spectrum of sunlight was first observed in 1951 (55). Previously, ultra violet light only had been implicated. A number of experiments were subsequently performed on colourless bacteria and fungi with carotenoid deficiencies resulting from genetic mutation or the inhibition of pigment synthesis by diphenylamine. In these it was demonstrated that a high light intensity was bacteriocidal. For example, a colourless mutant of S. lutea was photo-oxidised (53), and a culture of Cornybacterium poinsettiae was light sensitive during diphenylamine treatment, but sensitivity was lost when the inhibitor was removed (46). Further, when carotenoid-deficient, dark-grown cells of the fungus Dacrypinax spathularia, which can only synthesise carotenoid in the light, were exposed to sunlight death occurred. However, these unpigmented cells survived under anaerobic illumination (56).

Light of low intensity, coupled with an exogenous dye, was used in most other experiments. For example, toluidine blue facilitated the lethal photo-oxidation of colourless mutants of S. lutea (57) and diphenylamine inhibited cultures of Mycoplasma marinum (58) in an aerobic environment.

β-carotene protected mice against photosensitivity which had been induced by haematoporphyrin. It has therefore been suggested that carotenoids in the skin may offer protection against light (59).

(c) Role in Glucose Transport

Smith has postulated a mechanism whereby carotenoids located in the protoplast membrane of the pleuropneumonia-like organism Mycoplasma laidlawii strain B, could mediate both the uptake of glucose from the medium and the excretion of acetate (54). This theory is based on the observations that carotenoid formation varies with the concentration of lipids and glucose in the medium and that, in the absence of cholesterol, inhibition of carotenoid synthesis inhibits growth. Further, the neurosporol content is present as a glucoside and the stereo-configuration of neurosporol resembles that of ~~other~~ sterols supporting growth (60). The available evidence for this function is therefore circumstantial.

5. Carotenoids in Subcellular Particles- Mitochondria

(a) Presence in Mitochondria.

An early reference to the association of carotenoids with a mitochondrial preparation appeared in 1957 (61). During the preparation of tocopherol from beef heart mitochondria, Boumann and Slater mentioned that, following refluxing of an ethanolic extract in ascorbic acid and strong alkali, carotenoids and vitamin A were removed from the extract by separation on a Floridin XS Earth column. Two years later Green published the results of experiments in which three carotenoids were isolated from the electron transport particles of beef heart mitochondria (62). In the same year papers were published in which the carotenoid absorption maxima were shown to appear in the absorption spectrum of the neutral lipid extract of the succinic dehydrogenase complex of beef heart mitochondria, produced both by cholate and deoxycholate treatment (63, 64).

Jensen and Koford (65) observed a lipoprotein complex, containing carotenoids, associated with the mitochondrial fraction.

They suggest that this may have originated from soap micelle formation during neutralisation of the homogenate with KOH, or from the mitochondria during homogenisation. The latter was regarded as questionable, however, because of the low lipoprotein content. Alternatively, they suggest that the formation of these particles could represent the carotenoids isolated from mitochondria by Green and Basford.

A concept generally held by plant Biochemists is that carotenoids are restricted to the plastids and vacuoles of plants. Williams and Goodwin (66) examined the subcellular distribution of carotenoids in Paul's Scarlet Rose obtained by natural growth and tissue culture. In green tissues they found that almost all the carotenoid was attached to a particulate fraction and only trace amounts appeared in the centrifugal supernatant, whereas the tissue culture carotenoids were almost totally associated with the supernatant. Thus, in the absence of plastids capable of photosynthesis, carotenoids did not appear to be bound to subcellular particles.

Conversely, however, Crane [illegible] detected the association of carotenoids with plant mitochondria (67). To observe changes in the carotenoid content, they initially attempted to "clear" a mitochondrial suspension from cauliflower bud tissue with deoxycholate. The resultant suspension, retaining a residual turbidity, possessed an absorption spectrum which "appeared to represent carotenes". The authors make no comment on the implications of this observation, but it does not conclusively imply that the general concept is erroneous because the experimental conditions did not exclude the possibility of contamination by carotenoid from other subcellular sources.

(b) Role in Transport across Membranes.

The traditional view of the composition of many membranes surrounding subcellular particles, including mitochondria,

is that they are composed of two outer protein surfaces which encase an inner lipid layer.

Jahn (68) has proposed a theory for electronic conduction through membranes, associated with ion transport. He suggests that an oxidising/reducing system could exist on one protein surface releasing electrons during, for example, ATP hydrolysis. The presence of a conjugated lipid system existing transversely across the membrane could mediate the transfer of released electrons, by resonance, to the other protein surface where a further oxidising/reducing system might exist. The resulting e.m.f. developed could facilitate ion transport through the membrane pores. Lipid candidates suggested as the electron transporting agents are carotene, astacene, retinene, vitamin A, and crocetin. Thus, carotenoids could play a role in electron transport across membranes, such as that of the mitochondrion.

(c) Role in Electron Transport associated with Respiration.

Shifts in the absorption maxima of carotenoids during respiration, similar to those observed during photosynthetic electron transport, were noticed by Nishimira and Chance (48). Oxygenation of a Chromatium suspension resulted in changes in the carotenoid absorption spectrum, and during a steady state of respiration spectral changes were produced by antimycin A. As was mentioned on page 18, one cannot conclusively deduce from these observations that carotenoids participate in electron transport.

6. Conclusion

The association of carotenoids with certain tissues and subcellular structures, and theories concerning the function of these carotenoids have been discussed in this chapter. Evidence for the mechanism of carotenoid participation in all

these structures is inconclusive, and therefore all the theories based on this evidence are controversial.

The presence of carotenoids in subcellular fractions in which oxidative phosphorylation occurs, i.e. mitochondria, the bacterial protoplast membrane, and quantasomes, could implicate the involvement of carotenoids in this pathway. Their presence in the first particle is poorly documented, however, and therefore the initial aim of experimental work discussed in this thesis was to obtain more conclusive evidence of the presence of carotenoids in mitochondria from plant, animal, and microbial sources. Circumstantial evidence suggests that carotenoids may be associated with mitochondria from cauliflower buds and, more probably, from beef heart and therefore these tissues were examined, followed by an investigation of the carotenoid-containing yeast Rhodotorula rubra.

Following the establishment of the presence of carotenoids in mitochondria from one or all of these tissues, it was proposed that experiments should be devised to determine whether carotenoids participated in any physiological function of these mitochondria.

CHAPTER 2.

MATERIALS AND METHODS

1. General.
2. Fractionation Procedures for Cauliflower Buds and Stalks.
3. Fractionation Procedures for Heart Muscle.
4. Fractionation Procedures for Rhodotorula rubra.
5. Methods to Determine the Effect of Illumination on Cauliflower Bud Mitochondria.

1. General.

(a) Assay Methods.

(i) Succinoxidase.

Succinoxidase was assayed manometrically, at 25° C, in a medium composed of ~~1.0M~~ 0.1M sodium succinate, 0.04M phosphate buffer (pH 7.2), and 0.0001M $MgCl_2$. Activity was expressed as microlitres of oxygen utilised in unit time.

(ii) Succinic dehydrogenase.

The succinic dehydrogenase activity of heart muscle and cauliflower subcellular fractions was assayed at 37° C and 25° C respectively by the manometric, ferricyanide method of Aldridge and Johnson (69). Results were expressed as microlitres of carbon dioxide evolved in unit time.

(iii) Glucose-6-phosphatase.

Glucose-6-phosphatase activity was assayed by the method of Hulsman (70).

(iv) Protein.

Protein was estimated by the colourimetric method of Folin and Lowry (71).

(b) Extraction, Estimation, and Analysis of Carotenoids.

Redistilled solvents were used throughout.

(i) Extraction from buds, stalks, and subcellular fractions from cauliflowers.

Weighed preparations of lyophilised buds and stalks (cut as shown on page 31) and centrifugal fractions of cauliflowers were finely ground at room temperature with acid-washed sand and carotenoids were extracted with acetone. Bud and stalk extracts were dilute and were therefore concentrated by evaporation under nitrogen, and each extract was subsequently transferred to ether and then light petroleum (B. pt. 40-60° C) by the careful addition of tap water. (The pH of the distilled water available was sufficiently low to cause conversion of the 5-6 epoxy carotenoids to their 5-8 furanoid forms.) The extracts were then dried over anhydrous sodium sulphate and concentrated under nitrogen.

(ii) Estimation of pigment concentration.

Light absorption was measured by a Beckmann DB spectrophotometer with a one centimeter light path. This was equipped with a scale expander for linear recordings which was calibrated to give readings on a logarithmic scale.

The absorption spectra of the light petroleum extracts in 1ml. were used to calculate the carotenoid and chlorophyll content of unit dry weight. Total carotenoid was expressed in terms of the maximum optical density for the pigment mixture at about 440mμ. Absorbance at 660mμ was used to estimate the chlorophyll content.

The carotenoids extracted from subcellular fractions of cauliflower buds were contaminated with a material which had a high u.v. absorption extending into visible wavelengths. To obtain an estimate of the extinction due to carotenoids in these extracts, it was necessary to assess and subtract the contribution

of the contaminant to the absorption spectrum (see figure 1). Where relatively small concentrations of carotenoid were present, two extreme positions were drawn for the absorption of the contaminant and their mean calculated. The extinction used was that of the main maximum of the extract at approximately 440mμ .

(iii) Analysis of cauliflower carotenoids.

The stalk extract in light petroleum was saponified at room temperature for one hour by the addition of 10% KOH/methanol (v/v). Extracted bud carotenoids were not esterified and therefore saponification was omitted. Both extracts were partitioned between 95% methanol/5% water (v/v) and light petroleum, and the hypophasic carotenoids in the methanol layer were subsequently transferred to ether and then light petroleum. Chlorophyll, present only in the stalk extracts, separated in the hypophase and remained in the aqueous phase when carotenoids were transferred to light petroleum. Both epiphasic and hypophasic extracts in light petroleum were then washed with tap water, dried over anhydrous sodium sulphate, and concentrated under nitrogen.

Carotenoid extracts were analysed by column chromatography on calcium hydroxide/"hiflo supercel" (5/1,w/w) for the epiphasic pigments and magnesium oxide/"hiflo supercel" (2/1,w/w) or zinc carbonate for the hypophasic pigments. The series of eluting solvents used was a modification of that suggested by Karrer and Jucker (72) in which concentrations increasing from 0.5 to 15% of ether, acetone, ethanol and methanol in light petroleum (B.pt. 40-60° C) were used.

The carotenoids were characterised by comparison with known pigments as follows:-

(a) Position on columns.

(b) Absorption spectra.

(c) Thin layer chromatography. Epiphasic carotenoids were separated on keiselgel and hypophasic carotenoids on keiselguhr by the procedure

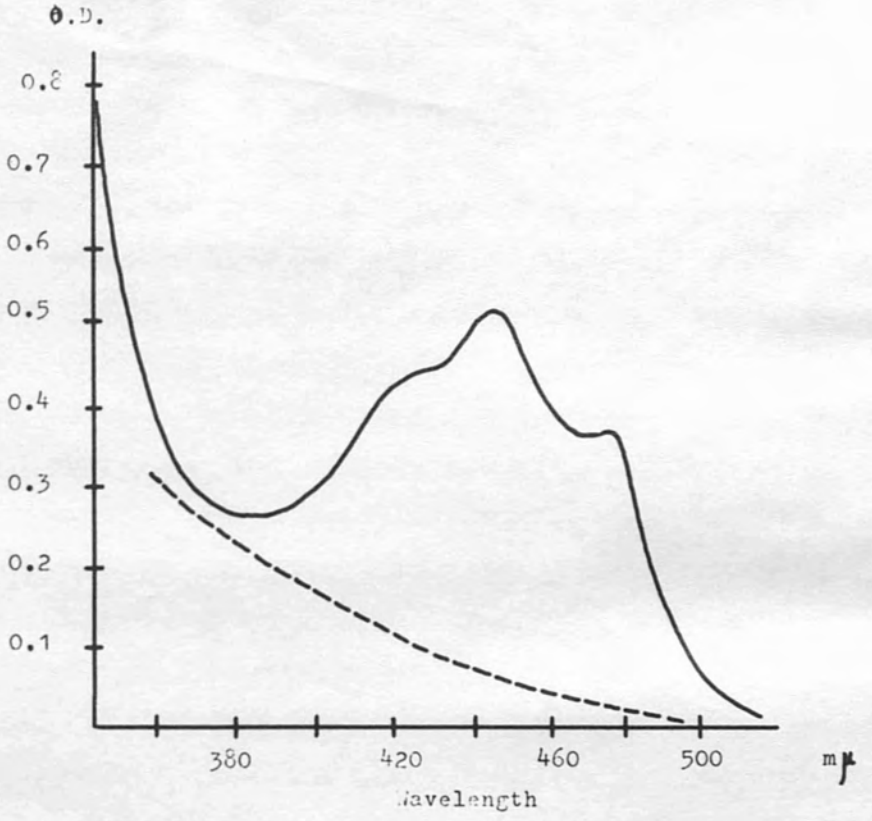


Figure 1. Typical absorption spectrum of acetone extract of centrifugal fraction of cauliflower bud homogenate, showing estimated position of absorbance due to the contaminant.

recommended by Randerath (73).

(d) Reaction with dilute acids.

(e) Partition coefficients. These were determined by Krinsky's method whereby carotenoids are partitioned between aqueous methanol and light petroleum (74).

(iv) Carotenoids of heart muscle tissue and centrifugal fractions.

The procedure for the extraction was an adaptation of the method described by Crane (75). 150gs. of heart muscle tissue was homogenised in a Waring blendor for five minutes with a solution of 10%KOH/methanol(w/v). Similarly, freeze-dried tissue or aqueous fractions, obtained by differential centrifugation of heart muscle homogenates, were added to alkaline methanol in a glass homogeniser. The suspensions were shaken for one hour, when carotenoid was extracted with ether, transferred to light petroleum, washed with tap water, partially evaporated in a stream of nitrogen to remove ether, and dried over anhydrous sodium sulphate. The maximum optical density at approximately 450mμ was obtained from the absorption spectrum of the extract. The constituent carotenoids were separated by the methods described in the previous paragraph for the carotenoids from buds and stalks.

(v) Carotenoids from ox blood.

Sodium oxalate was added to ox blood, obtained immediately after slaughter, to give a concentration of 4%, and this was diluted with one volume of water. The haemolysed blood was either added to 2 volumes of 10%KOH/methanol (w/v) or maintained at ambient temperature for two hours and then added to alkaline methanol. Following a saponification period of two hours, carotenoids were extracted with ether, transferred to light petroleum, washed, dried, and concentrated under nitrogen.

The carotenoid content was calculated from the maximum optical density at approximately 450mμ per ml. of blood.

2. Fractionation Procedure for Cauliflower Buds and Stalks.

(a) Cauliflower Bud and Stalk Tissue.

Cauliflowers, selected for their freshness, turgidity, and whiteness, were obtained throughout the year from Church Street market, Edgeware Road, London, N.W.1. They were used either immediately after purchase, or stored for one night at 0° C.

Bud tissue was cut from the top millimeter of the cauliflowers, every care being taken not to remove stalk tissue. The buds were collected in a beaker, surrounded by ice, and stored at 0° C for a period not exceeding two hours.

Stalk tissue, from the regions not immediately adjacent to the buds, was sliced thinly on to petri dishes placed on ice and stored in a manner similar to the buds.

(b) Homogenisation and Fractionation Procedures.

All procedures were carried out at 0° C unless otherwise stated.

(i) Homogenisation.

The blending medium in which cauliflower buds were homogenised contained 0.25M sucrose, 0.05M tris buffer (pH 7.2), 0.0001M EDTA, 5×10^{-3}M n-propyl gallate (NPG), and 5×10^{-3}M cysteine. (NPG and cysteine are used as inhibitors, see page 38.) 20g. batches of bud tissue were homogenised with 40ml. of blending medium, for 30 seconds, in an M.S.E. homogeniser with sharpened blades. A further 40ml. of blending medium was then added to the homogenate.

The stalk slices were homogenised, for 5 minutes, in a Waring blendor, with a solution containing 0.25M sucrose, 0.05M tris buffer (pH 7.2) and 0.0001M EDTA. Each suspension was then filtered through four layers of muslin.

(ii) Centrifugation.

The combined filtrate was fractionated by either differential centrifugation or discontinuous sucrose density gradient centrifugation (as shown in figure 2). In early experiments insufficient material was separated on the normal discontinuous gradient where all the solutions are present in one tube. The method was therefore modified so that the suspension was placed on one solution and was divided into two parts. These were subsequently sub-divided on other solutions of different molarities in separate tubes. By this modified method it was possible to fractionate larger quantities of homogenised tissue.

(iii) Division and washing of fractions.

The centrifugal pellets and material at the interfaces were evenly resuspended in a small quantity of blending medium, divided into two equal parts, and each suspension was diluted with the appropriate washing medium.

Suspensions to be analysed for carotenoid were washed twice, to remove sucrose, with the following medium:- 0.05M tris buffer (pH 7.2), $5x10^{-3}$M NPG and $5x10^{-3}$M cysteine. They were spun for thirty minutes at 38,000g after each washing and the final pellets were lyophilised, weighed, and carotenoids extracted and assayed by the procedure outlined on page 27.

For the assay of succinic dehydrogenase, the mitochondrial marker, a hypotonic medium was essential and therefore fractions to be assayed for this enzyme were diluted with 0.05M tris buffer (pH 7.2). (The inhibitors, NPG and cysteine, affected succinate oxidation and were therefore omitted from the medium.) The resultant pellets were resuspended in the same medium, left for one hour at 0^{o} C, resedimented, and the final pellets suspended in a small volume of a solution containing 2.5mM phosphate buffer (pH 7.4) and 4mM sodium succinate, as recommended by Johnson (76). The

Figure 2a. Scheme for the differential centrifugation of cauliflower buds and stalks.

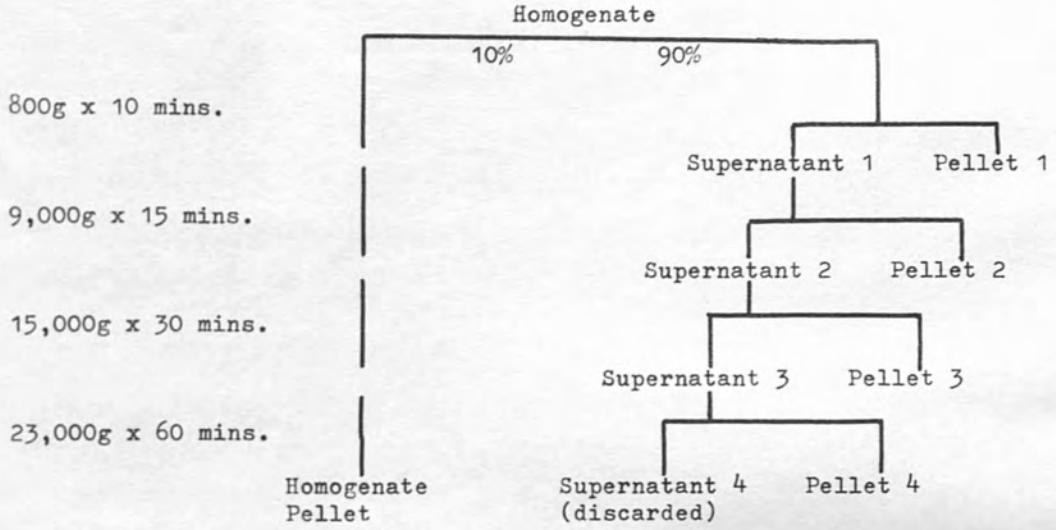
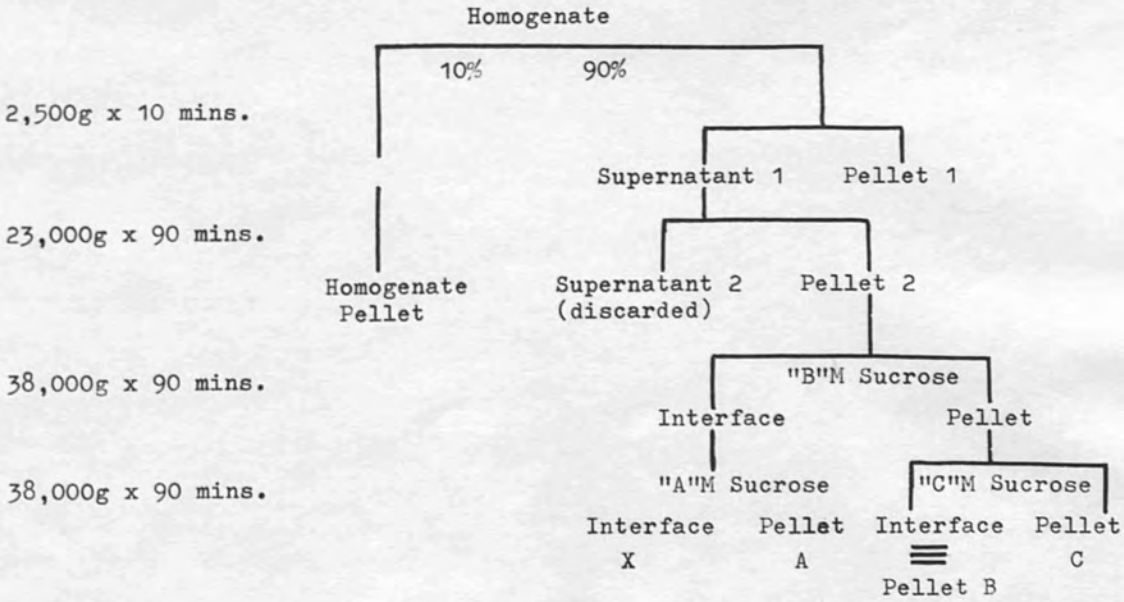


Figure 2b. Scheme for the discontinuous sucrose density gradient centrifugation of cauliflower bud homogenates.



Density of gradient solutions: C > B > A

final suspensions were frozen overnight, thawed the following day, and maintained at 0° C for a minimum period of one hour preceding the assay of succinic dehydrogenase (see page 26).

Succinoxidase was chosen as the mitochondrial marker in early experiments, when a half of each centrifugal fraction was assayed for this enzyme immediately following centrifugation. During subsequent density gradient separation succinoxidase was partially inactivated thus rendering the assay of this enzyme inaccurate as a mitochondrial marker. It was therefore replaced by succinic dehydrogenase.

(iv) Expression of results.

The carotenoid content and succinic dehydrogenase activity were expressed in terms of dry weight. The amount of material in the succinic dehydrogenase sample was equal to that of the carotenoid sample, the latter being lyophilised and weighed.

The recoveries of carotenoid, succinic dehydrogenase, and dry weight were expressed as a percentage of the total material which could be sedimented from the homogenate rather than as percentages of the content in whole buds or stalks. It was not possible to assay the enzymic activity and carotenoid content of the homogenate, which was extremely dilute. Further, experiments illustrated that carotenoid itself was more readily removed from homogenised tissue than whole tissue.

(c) Notes on the Development of the Procedure for Fractionation.

(i) Homogenisation of buds.

The method for the disruption of cellular material was based on the methods of Laties (77), Crane (67), and Lyons and Pratt (78). Of these papers, the most recent (78) recommended the use of 0.25M sucrose. Tris was found to be the most suitable

buffer, phosphate buffer having a deleterious effect on mitochondrial activity (79).

Two methods recommended for breaking the buds, hand grinding or homogenisation for five seconds, did not cause sufficient disruption. An experiment was therefore devised to observe the effect of increasing periods of homogenisation, using sharpened blades, on the activity of the mitochondria. Results of this experiment appear in Table 1.

Table 1. Succinoxidase activity of cauliflower bud suspensions resulting from different periods of homogenisation.

Length of homogenisation period (secs.)	20	40	60	90
Succinoxidase activity ($\mu l O_2$/min.)	0.69	0.7	0.35	0.24

20g. of bud tissue was homogenised for 20 seconds with 40 ml. of blending medium minus NPG and cysteine, using sharpened blades. After filtration an aliquot was removed and the suspension rehomogenised for further consecutive periods of 20, 20, and 30 seconds, an aliquot being removed following each homogenisation.

From these results it was decided that a homogenisation period of 30 seconds would not decrease the observed mitochondrial activity and subsequent experiments illustrated that this prolonged period of homogenisation increased the cellular disruption.

(ii) Effect of NPG.

Initially, the carotenoid extracted from centrifugal pellets was much smaller than was expected from comparisons with extracts of whole buds. This could have been caused by the association of the carotenoids with light particles remaining in the supernatant after centrifugation, inability to extract all the carotenoid in the pellets, or breakdown of carotenoids.

Free radical intermediates, formed during the oxidation of polyunsaturated fatty acids by the plant enzyme lipoxidase,

catalyse the oxidation of carotenoids and polyphenols (80). NPG is an inhibitor of this reaction. The presence of this enzyme was demonstrated by an experiment in which linoleate, but not oleate, was shown to be oxidised by a cauliflower bud extract (see table 2). The failure to oxidise oleate distinguishes lipoxidase from unspecific oxidation. This observation has been confirmed by O'Reilly and Prebble (81) who have partially purified the enzyme and determined its K_m.

Table 2. Lipoxidase activity of cauliflower bud homogenate.

Fatty acid assayed.	Linoleate	Linoleate	Linoleate	Linoleate	Oleate
Further additions	-	$5x10^{-3}$M NPG	Carotenoid	NPG and carotenoid	-
Total rate of Oxidation. ($\mu l O_2$/min./mg. protein	0.15	0	0.08	0	0

Lipoxidase activity of the cauliflower bud homogenate was assayed by the manometric method recommended by Tappel (82).

Inhibition of lipoxidase, by the addition of $5x10^{-3}$M NPG to the blending medium, increased the carotenoid extracted from sedimented cauliflower bud homogenates, but simultaneously increased the amount of u.v. absorbing material extracted. The absorption spectra of extracts obtained in a typical experiment, shown in figure 3, illustrate this conclusion.

Later experiments showed that the carotenoid from material recovered from media containing NPG was higher than that obtained by direct extraction of unhomogenised buds.

(ii) Effect of cysteine.

The u.v. absorbing material present in carotenoid extracts of centrifugal pellets was absent from extracts of whole buds and

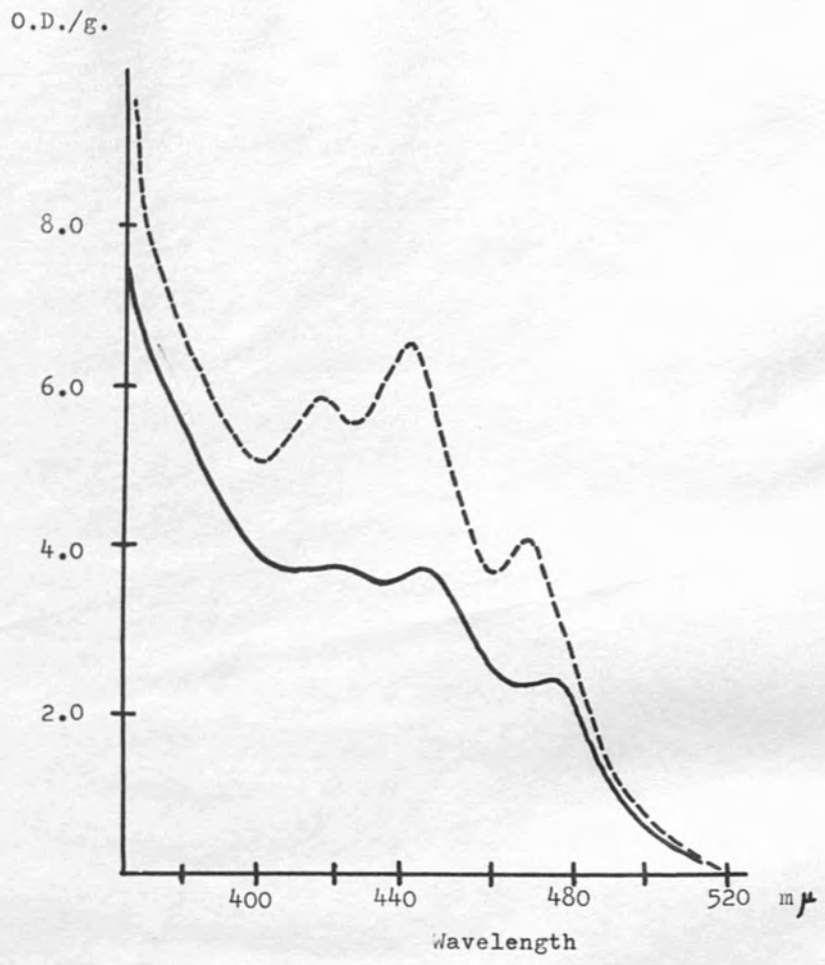


Figure 3. Absorption spectra of carotenoid extracted per g. dry weight in the absence and presence of $5x10^{-3}$M NPG.

—— buds homogenised for 30 secs. in 0.25M sucrose, 0.05M tris buffer (ph 7.2) and 0.0001M EDTA, incubated at 0°C for one hour, centrifuged at 23,000g for 30 mins. and lyophilised.

——— buds treated in a similar manner, but homogenisation medium contained $5x10^{-3}$M NPG.

was hence considered to have been produced following damage of the tissue. Cut cauliflower buds, when exposed to air, go brown in a manner resembling the effect of polyphenol oxidase on plant systems such as potatoes (83). On the assumption that this enzyme was responsible for producing the u.v. absorbing material, an experiment was performed in which cysteine ($5x10^{-3}M$), an inhibitor of polyphenol oxidase, was present in the blending medium. The results, shown in figure 4, illustrate that the presence of cysteine reduced the amount of u.v. absorption but simultaneously decreased the amount of carotenoid extracted.

(iv) Combined effect of NPG and cysteine.

The presence of $5x10^{-3}M$ NPG and $5x10^{-3}M$ cysteine in the blending medium resulted in an increase in carotenoid extracted and a decrease in u.v. absorbing material, as illustrated in table 3. Complete exclusion of the contaminant was not possible.

When stalk tissue was examined more carotenoid was extracted from the centrifugal fractions than from the unhomogenised tissue, suggesting a low level of lipoxidase in the stalks, and no u.v. absorbing material contaminated the acetone extracts. NPG and cysteine were therefore omitted from the blending medium.

(v) Effect of inhibitors on succinate oxidation.

Isolation of bud mitochondrial fractions in the presence of the inhibitors decreased succinate oxidation by about 70%. Attempts to compare the succinate oxidation of fractions separated in the absence of inhibitors with carotenoids separated in their presence were abandoned, however, as the inhibitors appeared to affect the distribution of the subcellular particles in the centrifugal fractions. Further, no basis could be found for comparison of the carotenoid content and succinate oxidation of equivalent fractions.

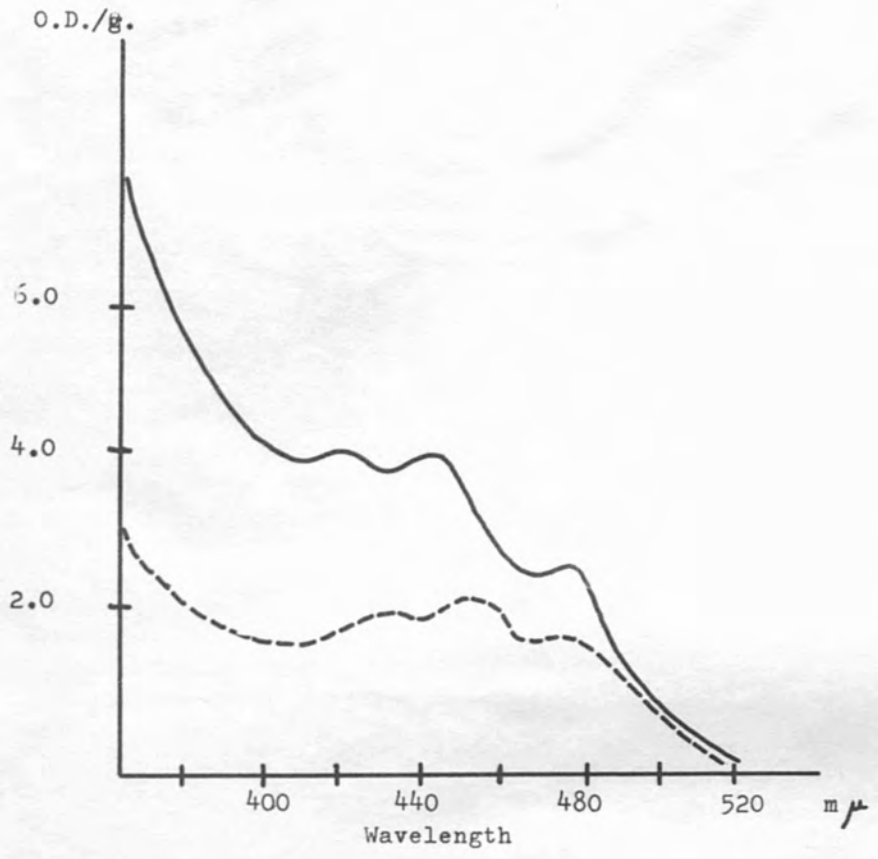


Figure 4. Absorption spectra of carotenoid extracted per g. dry weight in the absence and presence of $5x10^{-3}$M cysteine.

——— buds homogenised for 30 secs. in 0.25M sucrose, 0.05M tris buffer (pH 7.2) and 0.0001M EDTA, incubated at 0^{o}C for one hour, centrifuged at 23,000g for 30 mins. and lyophilised.

– – – buds treated similarly, but homogenisation medium contained $5x10^{-3}$M cysteine.

Table 3. Effect of NPG and cysteine on carotenoid and u.v. absorbing material of cauliflower bud homogenate extracts.

Addition to blending medium.	No addition	$5x10^{-3}$M NPG	$5x10^{-3}$M cysteine	NPG and cysteine	Whole buds
Carotenoid extracted/g. ($O.D._{440m}$ /g. D.W.)	2.14	5.35	1.4	5.7	3.6
u.v. absorbing material ($O.D._{270m}$ /g. D.W.)	2630	5000	749	1710	1420

A mixture of cauliflower buds was divided into 5 parts. One was lyophilised immediately and the others homogenised in the blending medium containing (i) no addition, (ii) $5x10^{-3}$M NPG, (iii) $5x10^{-3}$M cysteine, (iv) $5x10^{-3}$M NPG and $5x10^{-3}$M cysteine. Each homogenate was maintained at 0° C for one hour, spun at 23,000g for 30 minutes, and the resulting pellet lyophilised. Optical density at 270m of the acetone extracts of all pellets, transferred to light petroleum (see page 27) was recorded and the carotenoid assessed following subtraction from the observed spectrum of the estimated contaminant extinction.

3. Fractionation Procedures for Heart Muscle.

(a) Hearts.

Ox heart was obtained from the Chalfont Meat Company, Bootstridge Lane, Chalfont St Giles, Bucks., and pig hearts from T. Wall and Sons, (Meat and Handy Foods Ltd.) Atlas Road, London N.W.10. They were transported to the laboratory either at ambient temperature or surrounded by ice and water, and were then cleared of connective tissue, valves, and fat, sliced into small pieces, and stored at $-20^{o}C$.

When required, the tissue was partially thawed in water and ice, cut into thin slices, passed through a cooled mincing machine, and collected on ice.

(b) Washing of Minced Muscle and Preincubation with Blood.

The minced muscle was stirred vigorously with ice and cold tap water. Following sedimentation of the particulate material, the supernatant and lipid at the surface were discarded. Washings were repeated until the supernatant appeared free from haemoglobin, when the sediment was collected by filtration through gauze. In certain experiments the washing medium for the initial, final, and every third washing contained 0.2M KCN in 0.05 M tris buffer (pH 7.5) at $0^{o}C$.

In experiments where muscle was preincubated with haemolysed blood, a small volume of tap water was added to the unwashed minced muscle, mixed with ice, to haemolyse the blood corpuscles. The suspension was maintained in the presence of the released haemoglobin, at 0^{o} C, for either one or two hours, being agitated occasionally.

(c) Homogenisation and Differential Centrifugation.

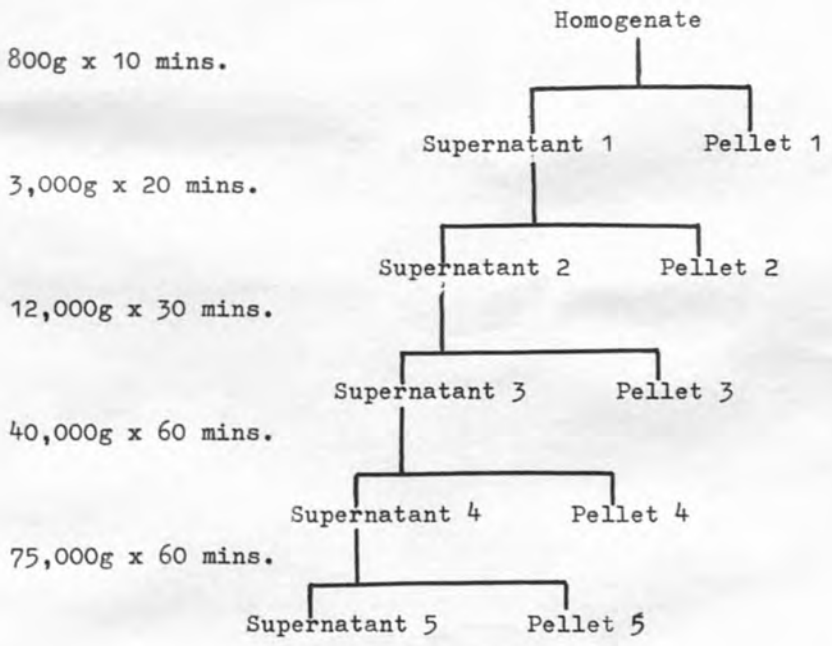
All procedures were performed at 0^{o} C unless otherwise stated.

(i) Homogenisation.

The blending medium consisted of 0.25M sucrose, 0.05M tris buffer (pH 7.2), 0.0001M EDTA, and in one experiment 0.2M KCN. 300 gs. of minced muscle was homogenised with 500 ml. of blending medium, in a Waring blendor, for 5 minutes, and the resulting suspension filtered through a series of gauzes composed of 1, 2, 4, and 6 layers of muslin.

(ii) The scheme used for the differential centrifugation of the heart muscle homogenate is shown in figure 5. Approximately 10% of the homogenate was retained, from which the percentages of carotenoid, succinic dehydrogenase, and protein recovered were calculated.

Figure 5. Scheme for the differential centrifugation of a heart muscle homogenate.



The homogenate sample and pellets separated before the termination of centrifugation were maintained in the dark at 0° C until the completion of fractionation, when all the pellets were evenly suspended in a small volume of the blending medium. All the suspensions, including the supernatant and the homogenate sample, were divided into two unequal parts of known volume in the ratio of approximately 1:6. The larger part was assayed for carotenoid content (see page 30), and the smaller was frozen overnight, thawed the following morning, maintained at 0° C for a minimum period of one hour and assayed for protein and succinic dehydrogenase activity. Carotenoid content and succinic dehydrogenase activity of the fractions were expressed in terms of their protein content.

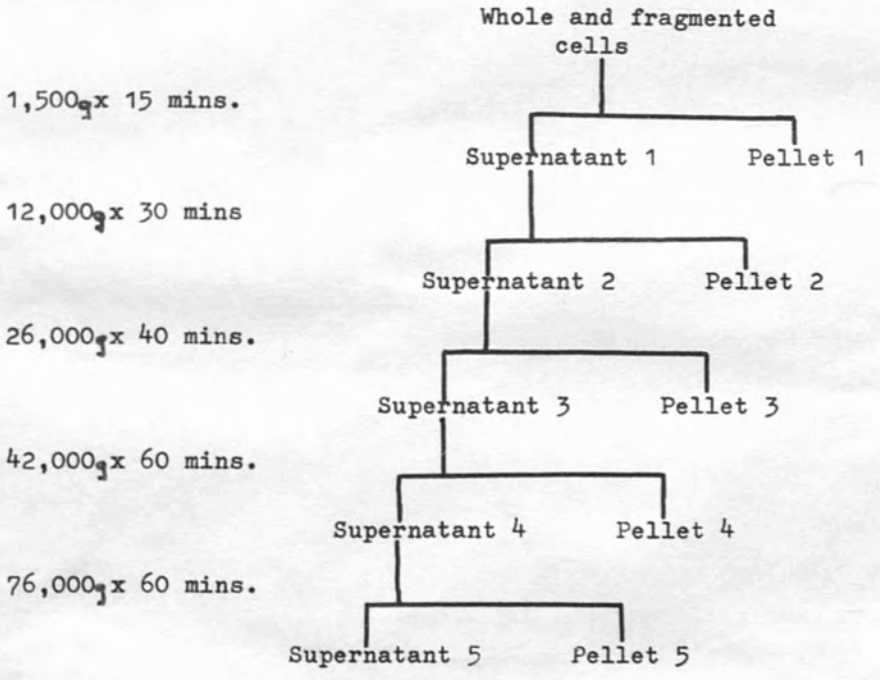
4. Fractionation Procedures for Rhodotorula rubra.

(a) Growth, Harvesting, and Fractionation of Cells.

Cultures of Rhodotorula rubra were grown on malt extract (Oxoid 57) at 26° C, in shake cultures, on agar plates, or in a fermenter. They were harvested by sedimentation at 1,500g for 10 minutes, washed with distilled water to remove the malt extract, and resuspended in the blending medium recommended by Cochrane (84). All subsequent procedures were performed at 0° C unless otherwise stated.

Maximum cell breakage (under 10%) was obtained by several passages of the culture through a modified French press (85) at pressures of 12-20,000 lb/sq.in. and the suspension so formed was fractionated by the scheme in figure 6.

Figure 6. Scheme for the differential centrifugation of Rhodotorula rubra.



The pellets were maintained at 0° C in the dark until centrifugation was completed when they were resuspended in a small volume of blending medium and divided into two unequal parts of known volume. The larger were assayed for carotenoid by the method of Peterson et al (97) and the smaller for succinoxidase (see page 26) and protein (see page 26). The carotenoid content and succinoxidase activity of the whole fractions were related to the protein content.

5. Methods to Determine the Effect of Illumination on Cauliflower Bud Mitochondria.

(a) Preparation of Mitochondrial Suspension.

Cauliflower bud tissue was homogenised by the procedure outlined on page 31 but the inhibitors were omitted from the blending medium. Following filtration a pellet, which sedimented by centrifugation at 1,500g for 10 minutes at 0° C, was discarded and the supernatant recentrifuged at 23,000g for 30 minutes. The resulting pellet, containing most of the mitochondria, was evenly resuspended in a small volume of supernatant or, alternatively, washed with a large volume of blending medium, resedimented, and dispersed in a small volume of the same medium at 0° C.

(b) Method of Assay of Succinoxidase.

The assay medium was identical to that described on page 26, 0.5ml. of the mitochondrial suspension being added to the Warburg flasks in random order. All flasks were duplicated with the exception of those used for the assay of endogenous activity, which was always extremely low. The suspension did not remain completely homogenous during the pipetting and therefore, to compensate for variations in the amount of preparation added, the initial rate of succinoxidase activity was recorded at intervals of 5 minutes, for 30 minutes, at 25° C, preceding illumination.

The perspex rotary Warburg bath (from B. Braun, Melsangen, West Germany) was illuminated by 14 x 40 watt tungsten bulbs producing a light intensity of 650 ft. candles at the base of each Warburg flask. Following the initial 30 minute assay the flasks were either illuminated or covered in aluminium foil to exclude all light and the manometers were read at intervals of 10 minutes for a period of up to 240 minutes.

The volume changes recorded by each manometer, when adjusted for endogenous activity, were corrected to give equal initial rates for comparable samples. The average value of the duplicates was subsequently calculated and, from these, comparisons could be drawn showing the effect of light on respiration. No effect of light was observed on endogenous activity.

(c) Supplementary Experimental Procedures.

(i) Preillumination.

In certain experiments the mitochondrial suspension was illuminated for 2 hours preceding enzyme assay. It was placed in a test tube in a water bath at 0° C and illuminated by 4 x 100 watt tungsten electric bulbs giving a light intensity of 650 ft. candles at the wall of the test tube. Heat from the bulbs was absorbed by a parallel-sided glass vessel, containing ice and water, placed 6 inches from both the bulbs and the water bath. Nitrogen or oxygen was slowly bubbled through the suspension to cause adequate stirring with minimal frothing.

(ii) Exogenous lipoxidase.

A preparation of the enzyme lipoxidase was added to certain Warburg flasks. The enzyme was prepared from soya bean meal by the method of Holman and Bergstrom (86). This procedure was followed to the stage where a precipitate, produced by ethanol fractionation, was redissolved in water and stored at 0° C. Crystallisation was not attempted.

0.1ml. of a 1:20 dilution of this enzyme preparation produced an increase in optical density of 0.0025 units / min. at 232mμ (due to linoleate oxidation) and a decrease of 6.7×10^{-6} units / min. at 440mμ (due to carotenoid oxidation) when incubated with 0.001M ammonium linoleate, 0.05M phosphate buffer (pH 7.2) and 0.2 ml. of a solution of cauliflower leaf carotenoids in ethanol/ acetone (50/50, v/v).

(iii) Exogenous Carotenoid.

The carotenoid solution, used in an attempt to protect mitochondrial carotenoids from oxidation caused by lipoxidase activity, was obtained by acetone extraction of cauliflower leaves (see method on page 27). The extract was saponified and transferred to light petroleum and therefore chlorophyll, which remained in the aqueous phase, was eliminated. This was then dried over sodium sulphate and evaporated to dryness under nitrogen. The deposit was dissolved in a solution composed of 50% acetone and 50% ethanol (v/v). From the absorption spectrum the optical density of this solution for a 1cm. light path was shown to be approximately 5.0. No material other than carotenoid, absorbing light between 340 and 500mμ, was detected. This indicated that the oxidised form of coenzyme Q, whose absorption spectrum in ethanol has a shoulder at about 420mμ, (87), was absent, although this did not exclude the presence of the reduced form which absorbs relatively little light in this region.

Chapter 3.

The Presence of Carotenoids in Cauliflower Bud Mitochondria

1. Introduction

Cauliflower buds, which are a good source of mitochondria and probably contain no plastids, were a convenient plant tissue in which to establish the presence of carotenoids in mitochondria. This series of experiments commenced with the extraction and analysis of bud carotenoids and their comparison with stalk carotenoids. Following the establishment of the presence of carotenoids in cauliflower buds, experiments were designed to determine whether carotenoids were located in the mitochondria.

Initial centrifugation experiments demonstrated the impossibility of exclusively restricting either carotenoids or mitochondria to one discrete fraction and therefore arbitrary fractions were collected. Assuming carotenoids to be located exclusively in the mitochondria, one would expect the sedimentation properties of carotenoid-containing particles and mitochondria to be identical. Likewise, the ratios of the carotenoid content and mitochondrial enzyme activity of all the fractions would be similar, within the limits of experimental error. The presence of carotenoids in particles other than, or as well as, mitochondria would be reflected in variations in these ratios. Attempts were therefore made to demonstrate the association of carotenoids with mitochondria by comparing the distribution of carotenoids and succinic dehydrogenase, the mitochondrial marker, in fractions obtained by differential

centrifugation and discontinuous sucrose density gradient separation.

For the purposes of comparison, two supplementary experiments were performed. In the first the distribution of carotenoid in centrifugal fractions of buds was compared with the distribution of glucose-6-phosphatase activity, which is frequently associated with the microsomal fraction. Secondly, the chlorophyll content of centrifugal fractions of cauliflower stalks was compared with their succinic dehydrogenase activity. Finally, to exclude the possibility of the presence of a carotenoid - containing particle with similar sedimentation properties to mitochondria, electron micrographs of centrifugal fractions were prepared and examined.

2. Analysis of the Carotenoid Content of Cauliflower buds

A number of experiments were carried out in which the carotenoid content of buds and stalks from the same cauliflowers were examined by the procedures described on pages 27 and 28 and the results of two of these experiments are shown in table 4. The groups of cauliflowers used in these experiments appeared to fall into two types, the carotenoid content in unit dry weight in the buds and stalks of one type being over twice as much as the other. However, this was the only difference detected between the groups of cauliflowers as the ratio of the epiphasic to hypophasic carotenoids in the buds and stalks was approximately constant and the composition of the extracts was similar in each experiment (see table 6).

Table 4. Total carotenoid and chlorophyll extracted from cauliflower buds and stalks.

Tissue	Buds	Buds	Stalks	Stalks
Experiment	A	B	A	B
Carotenoid content ($O.D._{440m\mu}/g.$)	7.2	18.8	0.114	0.37
% Total Carotenoid				
Epiphase	14%	13%	20%	18%
Hypophase	86%	87%	80%	82%
Chlorophyll content ($O.D._{660m\mu}/g.$)	0	0	3.5	3.2

For details of procedures for extraction and analysis, see pages 27 and 28.

There was clearly a difference in the carotenoid concentration and composition (in terms of epiphasic and hypophasic pigments) between the fully differentiated cells of the stalk and the largely meristematic and undifferentiated cells of the bud tissue.

The extracts were separated into their constituent carotenoids which were tentatively identified as β -carotene, lutein, violaxanthin, and neoxanthin from the properties listed in table 5. Thin layer chromatography of neoxanthin showed the presence of a contaminant which may have been responsible for the high M_{50} observed.

The average quantitative composition of the carotenoid extracts, expressed as a percentage of the total weight of carotenoid, is compared to the carotenoid contents of light- and dark- grown wheat (90) and spinach quantasomes (91) in table 6.

Table 5. Identification of cauliflower bud carotenoids.

Probable identification	Eluting solvent in light petroleum			Spectral maxima in light petroleum	M_{50} (74)		Effect of dilute acids
	Mg_2O/ hiflo supercel 2:1	$ZnCO_3$	$Ca(OH)_2$/ hiflo supercel 5:1		Sample	Standard	Product Identification
NEOXANTHIN (88)	2% ethanol	3% ethanol		441-443mμ 471-473mμ	52	45	max. 402mμ 422-425mμ 449-451mμ M_{50} 58
VIOLAXANTHIN	10% acetone (89)	10% acetone		419.5mμ 442-443.5mμ 472-473.5mμ	68	67	max. 382mμ 405mμ 430mμ M_{50} 66 Auroxanthin
LUTEIN	1% ethanol (89)	5% acetone		445-447mμ 475-477mμ	83.5	82	-ve.
β -CAROTENE			1 % ether	450.5mμ 479mμ			-ve.

Table 6. Comparison of the composition of carotenoid extracts.

Pigment	$E^{1\%}_{1cm.}$ (92)	% of Total Carotenoids				
		Bud	Stalk	Wheat (90) Light grown	Wheat (90) Dark grown	Quantasome (91)
Neoxanthin	2270	10.5	21	3.3	13	11.8
Unidentified xanthophyll				7.2	15.7	
Violaxanthin	2216	44.5	14.5	3.2	6.5	14
Lutein epoxide				12.9	12	
Neozeaxanthin					9.5	
Lutein	2160	33	45.5	39	35.4	45.5
Carotenes	2505	12	21	34	7.6	28.7

The carotenoids present in cauliflower buds and stalks were similar and resembled those in wheat and quantasomes. However, violaxanthin was the major constituent of the bud extract, followed by lutein, the carotenes, and finally neoxanthin whereas in the other extracts, all of which were obtained from chlorophyll-containing tissues, lutein was the major constituent followed by the carotenes and the proportion of violaxanthin was much lower than in the buds.

From these results it was established that carotenoids were present in cauliflower bud tissue. The investigation was therefore continued in an attempt to ascertain the subcellular location of carotenoids in this tissue.

3. Centrifugal Fractionation

(a) Initial Experiments

Initial attempts to fractionate cauliflower bud homogenates by differential centrifugation demonstrated that neither carotenoid nor the ability to oxidise succinate was restricted to one discrete centrifugal fraction. This fact is illustrated by the results of an early experiment, which appear in table 7. In this experiment the carotenoid content of centrifugal fractions, separated in a medium containing NPG and cysteine (by the procedures outlined on pages 31 to 34), was compared with succinoxidase activity of fractions separated simultaneously in the absence of the inhibitors (which inhibit succinoxidase activity).

The carotenoid content of the fractions was expressed in terms of dry weight and the succinoxidase activity in terms of protein. However, the ratio of the protein content to dry weight varied between the fractions, as table 7 illustrates, and therefore comparisons between the carotenoid content/g. dry weight and succinoxidase activity / mg. protein of equivalent fractions were meaningless. It was not feasible to compare the carotenoid content and succinoxidase activity of equivalent fractions as changes may have occurred in the way in which particles containing the substances were distributed. For example, the inhibitors may have affected the density and weight of the particles. However, similarities in the ratio of the carotenoid content to succinoxidase activity in equivalent fractions suggested that particles containing these two substances may have followed the same sedimentation patterns.

(b) Differential Centrifugation

In subsequent experiments the basic procedure outlined on pages 31 to 34 was followed, all centrifugal pellets, separated in the presence of the inhibitors, being divided into two equal parts which were assayed for carotenoid content and succinic

Table 7. Examination of carotenoid content of fractions of cauliflower bud tissue separated by differential centrifugation in an inhibited medium and the succinoxidase activity of fractions separated in the absence of the inhibitors.

	Homogenate sample	Pellet 1	Pellet 2	Pellet 3	Pellet 4
Carotenoid content ($O.D._{440m\mu}$)					
Estimated min. value	0.27	1.17	0.48	0.28	0.17
Estimated max. value	0.29	1.30	0.51	0.31	0.20
Average value	0.28	1.23	0.50	0.30	0.19
Dry weight (g.)	0.047	0.245	0.042	0.025	0.025
Average carotenoid content/g. dry weight	6.1	5.0	11.9	11.9	7.6
Succinoxidase activity ($\mu l.O_2$/min.)	0.26	0.95	0.51	0.25	0.13
Protein content (g.)	0.022	0.051	0.030	0.010	0.008
Succinicoxidase activity/ g. protein	11.6	18.6	17	25	15
Ratio $\frac{\text{Average Carotenoid content}}{\text{Succinoxidase activity}}$	1.13	1.28	0.97	1.21	1.36
Ratio $\frac{\text{Average Carotenoid/g. dry weight}}{\text{Succinoxidase activity /g. protein}}$	0.54	0.27	0.70	0.47	0.50
Ratio $\frac{\text{Dry weight (g.)}}{\text{Protein (g.)}}$	2.1	4.8	1.38	2.5	2.82

Percentage Recoveries:- Carotenoid 87% Succinoxidase activity 81%
Dry Weight 80% Protein 103%

dehydrogenase activity. Both properties were expressed in terms of the dry weight of the carotenoid sample. Succinoxidase was replaced as the mitochondrial marker by succinic dehydrogenase which did not appear to be inactivated by concentrated sucrose solutions.

The results of a representative experiment in which fractions were separated by differential centrifugation by the modified method, appear in table 8. These results illustrate that the centrifugal pellets 1 and 3 contained respectively the highest and second highest amounts of both carotenoid and succinic dehydrogenase activity, but the third fraction contained the greatest concentration of both carotenoid and succinic dehydrogenase activity. When the amount of carotenoid in the fractions was plotted against their succinic dehydrogenase activity, a linear relationship was observed (see figure 7). Similarly, the ratios of the carotenoid content and succinic dehydrogenase activity of the fractions were alike, the standard deviation in the ratios of 14.7% being taken to represent experimental error and limitations in the method. Therefore, it was concluded that the sedimentation patterns of particles containing carotenoid and succinic dehydrogenase activity were similar.

In all similar experiments in which cauliflower bud tissue was fractionated by differential centrifugation a correlation was invariably obtained between the distribution of carotenoid and succinic dehydrogenase activity in the fractions. Differential centrifugation was considered to be a fairly coarse method by which to demonstrate the association of carotenoids with mitochondria however and therefore separation using a discontinuous sucrose density gradient was attempted.

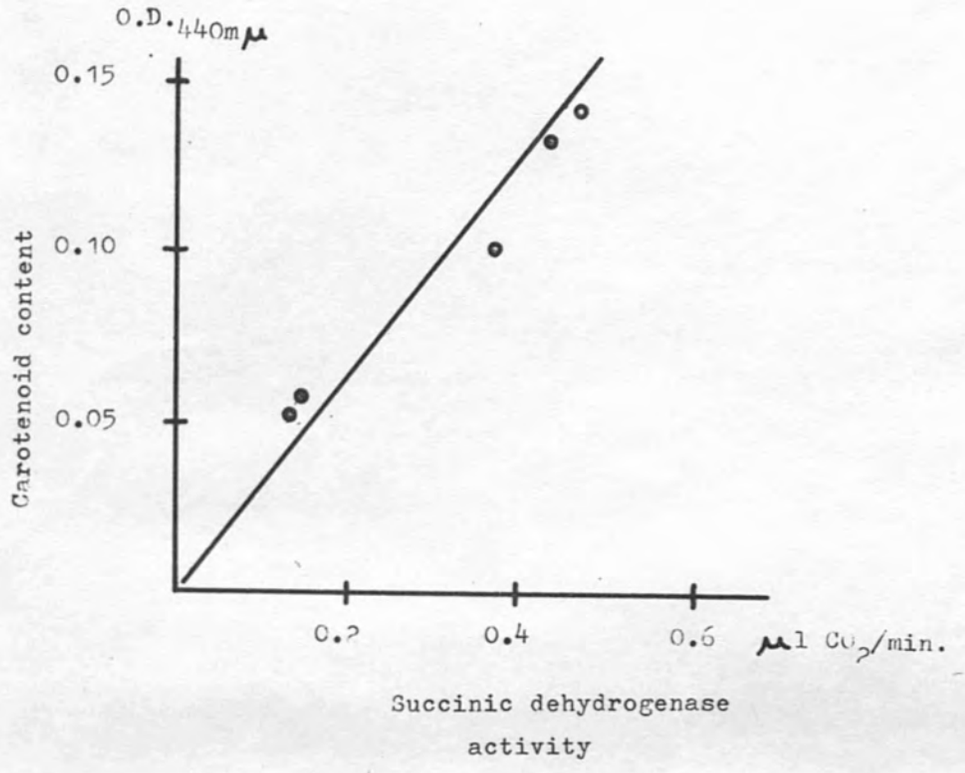


Figure 7. Comparison of the carotenoid content and succinic dehydrogenase activity of fractions of a cauliflower bud homogenate separated by differential centrifugation. (see table 8.)

Table 8. Analysis of fractions of a cauliflower bud homogenate separated by differential centrifugation.

For details of homogenisation, differential centrifugation, division and washing of fractions, and the assay of carotenoid content and succinic dehydrogenase activity, see pages 31 to 34.

Table 8.

	Homogenate sample	Pellet 1	Pellet 2	Pellet 3	Pellet 4
Carotenoid content ($O.D._{440m\mu}$)					
Estimated max. value	0.067	0.122	0.110	0.162	0.057
Estimated min. value	0.050	0.159	0.090	0.112	0.045
Average value	0.058	0.140	0.100	0.137	0.051
Succinic dehydrogenase activity ($\mu l.CO_2$/min.)	0.148	0.469	0.365	0.437	0.136
Dry weight (g.)	0.057	0.250	0.124	0.033	0.018
Average Carotenoid/ g. dry weight	1.02	0.56	0.85	5.25	2.77
Succinic dehydrogenase activity/g. Dry weight	2.6	1.94	3.05	13.4	7.4
Ratio $\frac{\text{Average Carotenoid content}}{\text{Succinic dehydrogenase activity}}$	0.40	0.30	0.27	0.31	0.38

Mean ratio $\frac{\text{Average Carotenoid content}}{\text{Succinic dehydrogenase activity}}$ 0.33

Standard deviation 14.7%

Percentage recoveries:-

Carotenoid	84%
Succinic dehydrogenase activity	105%
Dry weight	84%

(c) Discontinuous Sucrose Density Gradient Centrifugation.

To determine the molarity of solutions which had densities similar to those of mitochondria, initial fractionations were carried out on a course discontinuous sucrose density gradient in which adjacent solutions differed by 0.3M sucrose (for details of fractionation procedure, see pages 31 - 34). The results of a typical experiment appear in table 9 and figure 8, and show that the majority of carotenoid and mitochondrial enzyme activity were in particles with densities equivalent to 1.0 to 1.6M sucrose. The sensitivity of the gradient was then increased by using a series of three solutions differing by intervals of 0.15M sucrose. The results (table 10, figure 9) showed that particles containing carotenoid and succinic dehydrogenase activity had densities equivalent to 1.1 to 1.4M sucrose. In both these experiments the particles containing carotenoid and succinic dehydrogenase activity appeared to have similar density distributions. There was a linear relationship between the carotenoid content and succinic dehydrogenase activity of the fractions (see figures 8 and 9), and the ratios of these perameters in the fractions were similar in each experiment, having standard deviations of 12% in the first gradient experiment described and 7.5% in the second. Therefore carotenoid and succinic dehydrogenase activity were distributed throughout the fractions in the same way.

Subsequent attempts to increase the sensitivity of the gradient were limited by two factors. Firstly, there appeared to be two regions on the gradient where the majority of carotenoid-containing particles and succinic dehydrogenase activity were located, being separated by a density approximately equivalent to 0.25M sucrose. The proportions of particles in these two regions varied with the sharpness of the blades of the homogeniser and the turgidity of the cauliflowers, neither of which could be adequately controlled. This suggests that

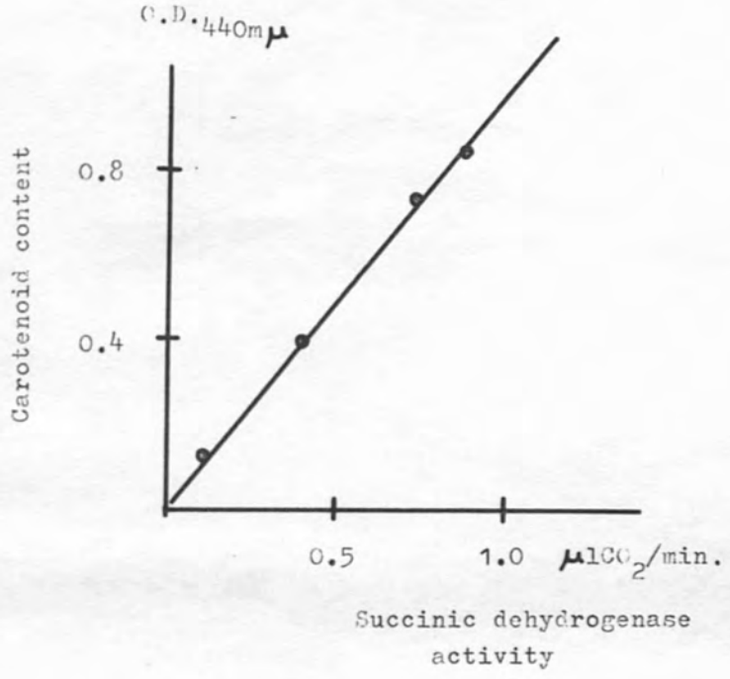


Figure 8. Comparison of the carotenoid content and succinic dehydrogenase activity of fractions of a cauliflower bud homogenate separated on a gradient composed of 1.0, 1.3, and 1.6M sucrose. (see table 9)

Table 9. Analysis of fractions of a cauliflower bud homogenate separated on a gradient composed of 1.0, 1.3, and 1.6M sucrose.

For details of homogenisation procedure, differential and gradient centrifugation, nomenclature of fractions, division and washing of pellets, and the assay of carotenoid content and succinic dehydrogenase activity, see page 31 to 34.

Table 9.

	Homogenate sample	Pellet 1	Pellet X	Pellet 1.0	Pellet 1.3	Pellet 1.6
Carotenoid content ($O.D._{440m\mu}$)						
Estimated min. value	0.39	0.796	0.058	0.75	0.122	0.023
Estimated max. value	0.42	0.900	0.048	0.71	0.133	0.030
Average value	0.40	0.848	0.053	0.73	0.127	0.027
Succinic dehydrogenase activity ($\mu l.CO_2$/min.)	0.385	0.87	0	0.76	0.105	0
Dry weight (g.)	0.083	0.201	0.005	0.094	0.017	0.004
Average Carotenoid/ g. dry weight	4.8	4.1	9.8	7.7	7.3	6.4
Succinic dehydrogenase activity/g. dry weight	4.7	4.3	0	8.1	6.1	0
Ratio $\frac{\text{Average Carotenoid content}}{\text{Succinic dehydrogenase activity}}$	1.05	0.94	-	0.97	1.22	-

Mean ratio $\frac{\text{Average Carotenoid content}}{\text{Succinic dehydrogenase activity}}$ = $\underline{\underline{1.05}}$

Standard deviation 12%

Percentage Recoveries:-

Carotenoid	90%
Succinic dehydrogenase activity	80%
Dry weight	78%

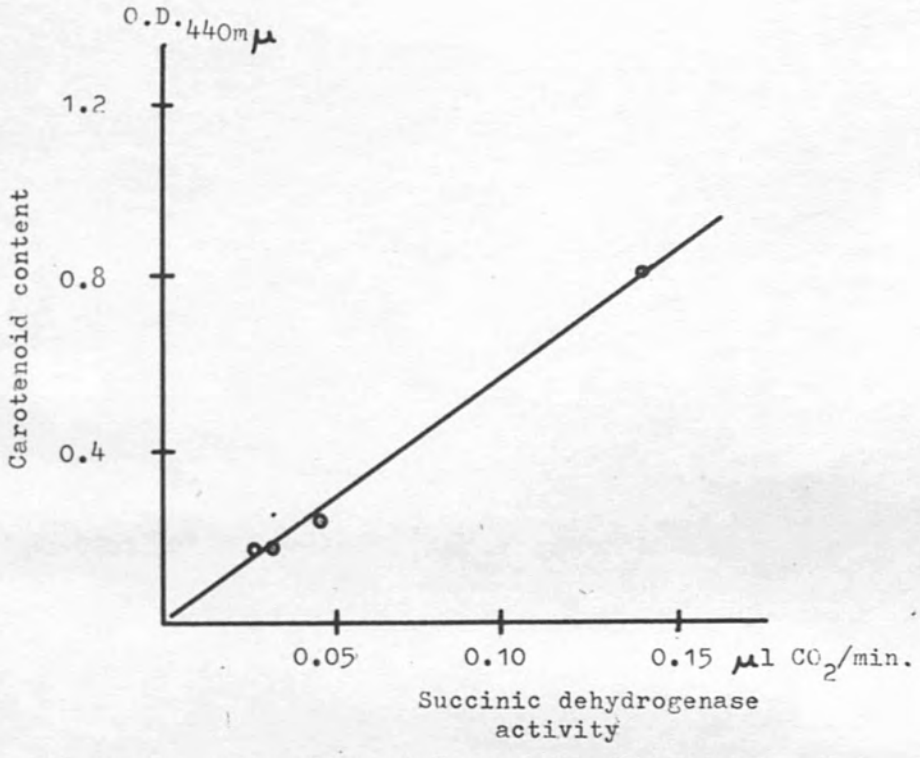


Figure 9. Comparison of the carotenoid content and succinic dehydrogenase activity of fractions of cauliflower bud tissue separated on a gradient composed of 1.1, 1.25, and 1.4M sucrose. (see table 10)

Table 10. Analysis of fractions of cauliflower bud tissue separated on a gradient composed of 1.1, 1.25, and 1.4M sucrose.

For details of homogenisation, differential centrifugation and gradient centrifugation, division and washing of fractions, and assay of carotenoid and succinic dehydrogenase activity, see page 31 to 34.

Table 10.

	Homogenate sample	Pellet 1	Pellet X	Pellet 1.1	Pellet 1.25	Pellet 1.4
Carotenoid content ($O.D._{440m\mu}$)						
Estimated min. value	0.159	0.81	0.039	0.230	0.171	0.028
Estimated max. value	0.195	0.84	0.039	0.244	0.182	0.032
Average value	0.175	0.82	0.039	0.237	0.176	0.030
Succinic dehydrogenase activity ($\mu l.CO_2$/min.)	0.029	0.142	0	0.046	0.030	0
Dry weight (g.)	0.047	0.207	0.008	0.037	0.062	0.004
Average Carotenoid/ g. dry weight	3.8	4.0	4.7	6.4	2.8	6.7
Succinic dehydrogenase activity/g. dry weight	0.62	0.67	0	1.25	0.49	0
Ratio $\frac{\text{Average Carotenoid content}}{\text{Succinic dehydrogenase activity}}$	6.1	5.7	-	5.2	5.8	-

Mean ratio $\frac{\text{Average Carotenoid content}}{\text{Succinic dehydrogenase activity}}$ 5.7

Standard deviation 7.5%

Percentage recoveries:-

Carotenoid	80%
Succinic dehydrogenase activity	68%
Dry weight	75%

one area may have contained broken particles. However, secondly, a total dry weight of 0.02 g. in each fraction was essential for the accurate assay of succinic dehydrogenase activity and carotenoid.

Because of these limitations it was not possible to separate the homogenate particles on gradients in which the adjacent solutions differed by intervals less than 0.1M sucrose. However, it was possible to perform a series of experiments in which the average molarity of the gradient solutions differed by 0.05M sucrose, but adjacent solutions on the gradient differed by 0.1M sucrose. This would therefore be equivalent to placing frontiers on the gradient at intervals of 0.05M sucrose and would effectively extend the number of solutions forming the gradient. A series of experiments arranged in this manner was therefore carried out but an additional experiment was performed in which the solutions on the gradient differed by 0.075M sucrose. The results of these experiments, in which the sucrose molarities used ranged from 1.10 to 1.45, are shown in tables 11 to 14 and graphically in figures 10 to 13. In certain fractions succinic dehydrogenase activity was not detected. One cannot assume that mitochondria were absent from these fractions, however, because the enzyme activity was reduced by NPG and cysteine in the blending medium, thus reducing the sensitivity of the assay.

In all the experiments the fraction containing the most carotenoid also possessed the greatest succinic dehydrogenase activity; the carotenoid content of each fraction appeared to be a function of the succinic dehydrogenase activity; and the standard deviation from the mean ratio of these perameters in each experiment ranged from 15.4 to 2.4%. The percentage recoveries of carotenoid, succinic dehydrogenase activity,

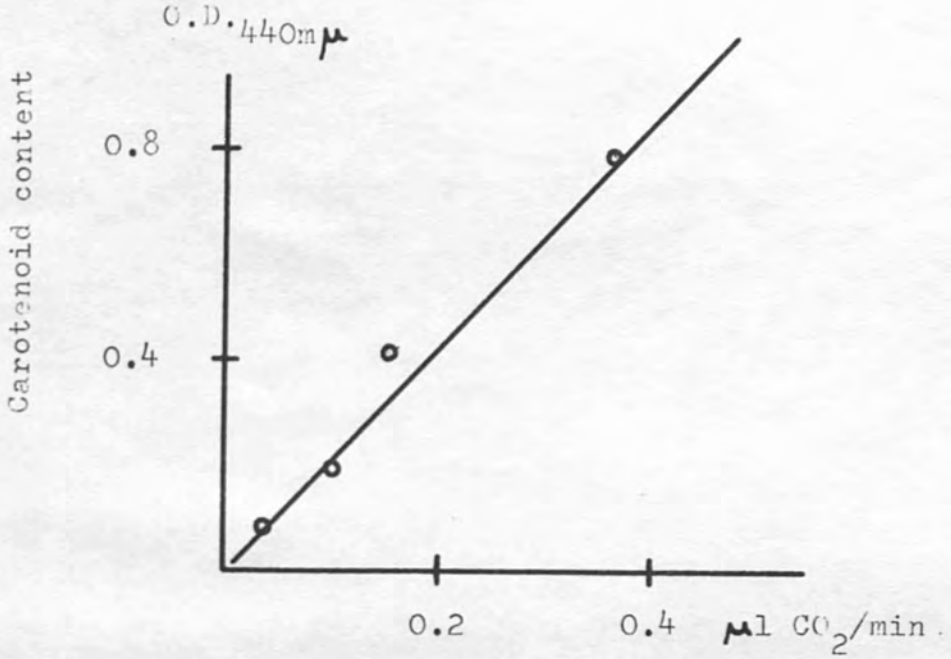


Figure 10. Comparison of the carotenoid content and succinic dehydrogenase activity of fractions of a cauliflower bud homogenate separated on a gradient composed of 1.1, 1.175, and 1.25M sucrose. (see table 11)

Table 11. Analysis of fractions of a cauliflower bud homogenate separated on a gradient composed of 1.1, 1.175, and 1.25M sucrose.

For details of homogenisation procedure, differential and gradient centrifugation, nomenclature of fractions, division and washing of pellets, and the assay of carotenoid content and succinic dehydrogenase activity, see page 31 to 34.

Table 11.

	Homogenate sample	Pellet 1	Pellet X	Pellet 1.1	Pellet 1.175	Pellet 1.25
Carotenoid content ($O.D._{440m\mu}$)	0.193	0.785	0.114	0.038	0.079	0.42
Succinic dehydrogenase activity ($\mu l.CO_2$/min.)	0.101	0.368	0	0	0.039	0.157
Dry weight (g.)	0.076	0.290	0.023	0.007	0.034	0.139
Carotenoid content/ g. dry weight	2.89	2.69	4.96	5.29	2.33	3.01
Succinic dehydrogenase activity/g. dry weight	1.34	1.27	0	0	1.01	1.13
Ratio $\frac{\text{Carotenoid content}}{\text{Succinic dehydrogenase activity}}$	1.92	2.13	-	-	2.04	2.6

Mean ratio $\frac{\text{Carotenoid content}}{\text{Succinic dehydrogenase activity}}$ 2.17

Standard deviation 13.6%

Percentage recoveries:-

Carotenoid	83%
Succinic dehydrogenase activity	64%
Dry weight	73%

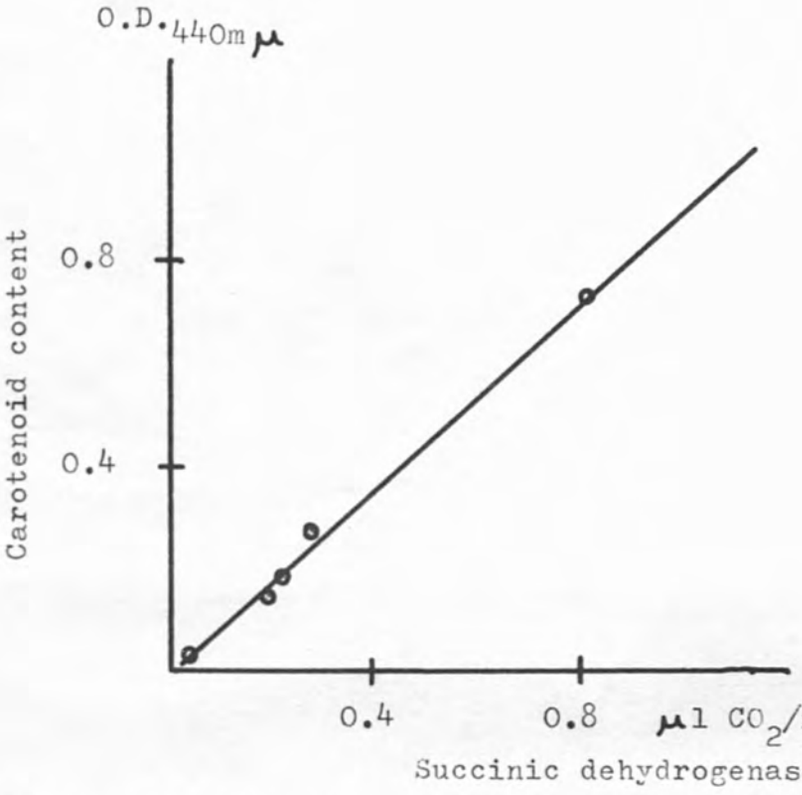


Figure 11. Comparison of the carotenoid content and succinic dehydrogenase activity of fractions recovered from a sucrose gradient composed of 1.15, 1.25, and 1.35M sucrose.(see table 12)

Table 12. Analysis of fractions of cauliflower bud tissue separated on a gradient composed of 1.15, 1.25, and 1.35M sucrose.

For details of homogenisation, differential and gradient centrifugation, nomenclature of fractions, division and washing of fractions and assay of carotenoid content and succinic dehydrogenase activity, see page 31 to 34.

Table 12

	Homogenate sample	Pellet 1	Pellet X	Pellet 1.15	Pellet 1.25	Pellet 1.35
Carotenoid content ($O.D._{440m\mu}$)						
Estimated min. value	0.156	0.67	0.142	0.024	0.270	0.184
Estimated max. value	0.178	0.82	0.158	0.040	0.295	0.206
Average value	0.167	0.74	0.150	0.031	0.282	0.195
Succinic dehydrogenase activity ($\mu l.CO_2$/min.)	0.183	0.81	0.195	0.034	0.272	0.217
Dry weight (g.)						
Average Carotenoid/ g. dry weight	3.38	2.59	6.55	2.12	3.77	2.89
Succinic dehydrogenase activity/g. dry weight	3.66	2.96	8.4	2.26	3.6	3.2
Ratio $\frac{\text{Average Carotenoid content}}{\text{Succinic dehydrogenase activity}}$	0.91	0.92	0.77	0.93	1.03	0.91

Mean ratio $\frac{\text{Average Carotenoid content}}{\text{Succinic dehydrogenase activity}}$ = 0.91

Standard deviation = 9.1%

Percentage recoveries:-

Carotenoid	94%
Succinic dehydrogenase activity	93%
Dry weight	98%

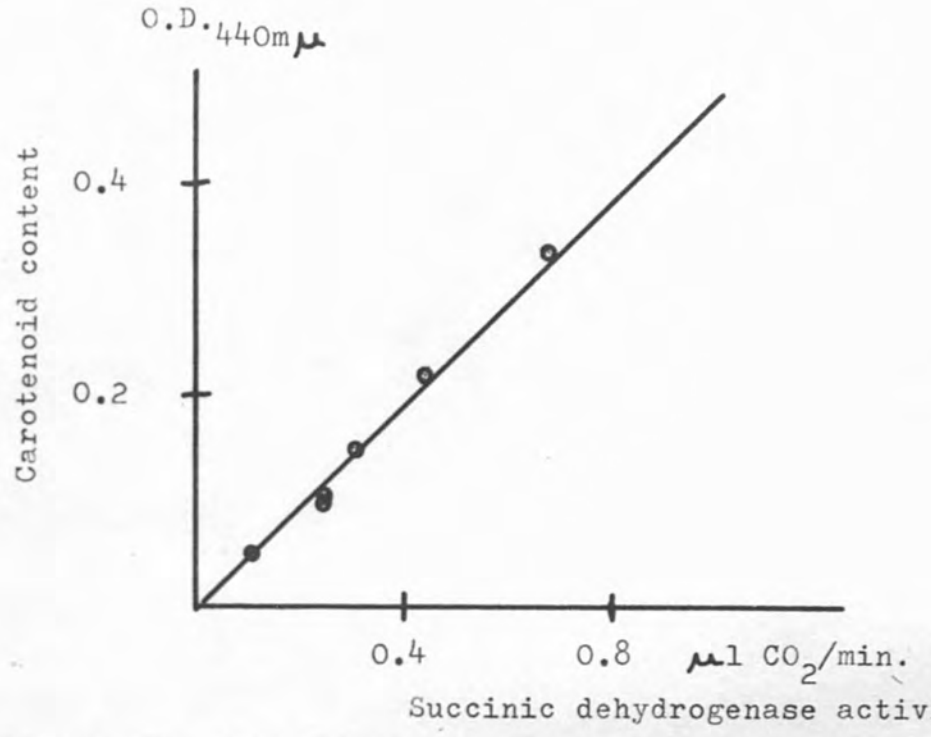


Figure 12. Comparison of the carotenoid content and succinic dehydrogenase activity of fractions of a cauliflower bud homogenate separated on a gradient composed of 1.2, 1.3, and 1.4M sucrose. (see table 13)

Table 13. Analysis of fractions of cauliflower bud tissue separated on a gradient composed of 1.2, 1.3, and 1.4M sucrose.

For details of homogenisation, differential and gradient centrifugation, nomenclature of fractions, division and washing, and the assay of carotenoid content and succinic dehydrogenase activity, see pages 31 to 34.

Table 13.

	Homogenate sample	Pellet 1	Pellet X	Pellet 1.2	Pellet 1.3	Pellet 1.4
Carotenoid content ($O.D._{440m\mu}$)	0.109	0.34	0.099	0.054	0.218	0.154
Succinic dehydrogenase activity ($\mu l.CO_2/min.$)	0.24	0.67	0.24	0.11	0.44	0.30
Dry weight (g.)	0.046	0.235	0.039	0.016	0.018	0.039
Carotenoid content/ g. dry weight	2.7	1.4	2.6	3.3	1.2	3.9
Succinic dehydrogenase activity/g. dry weight	5.3	2.5	6.2	6.8	2.4	7.7
Ratio $\frac{\text{Carotenoid content}}{\text{Succinic dehydrogenase activity}}$	0.45	0.51	0.42	0.48	0.49	0.51

Mean ratio $\frac{\text{Carotenoid content}}{\text{Succinic dehydrogenase activity}}$ = 0.48

Standard deviation = 2.4%

Percentage recoveries:-

Carotenoid	87%
Succinic dehydrogenase activity	81%
Dry weight	84%

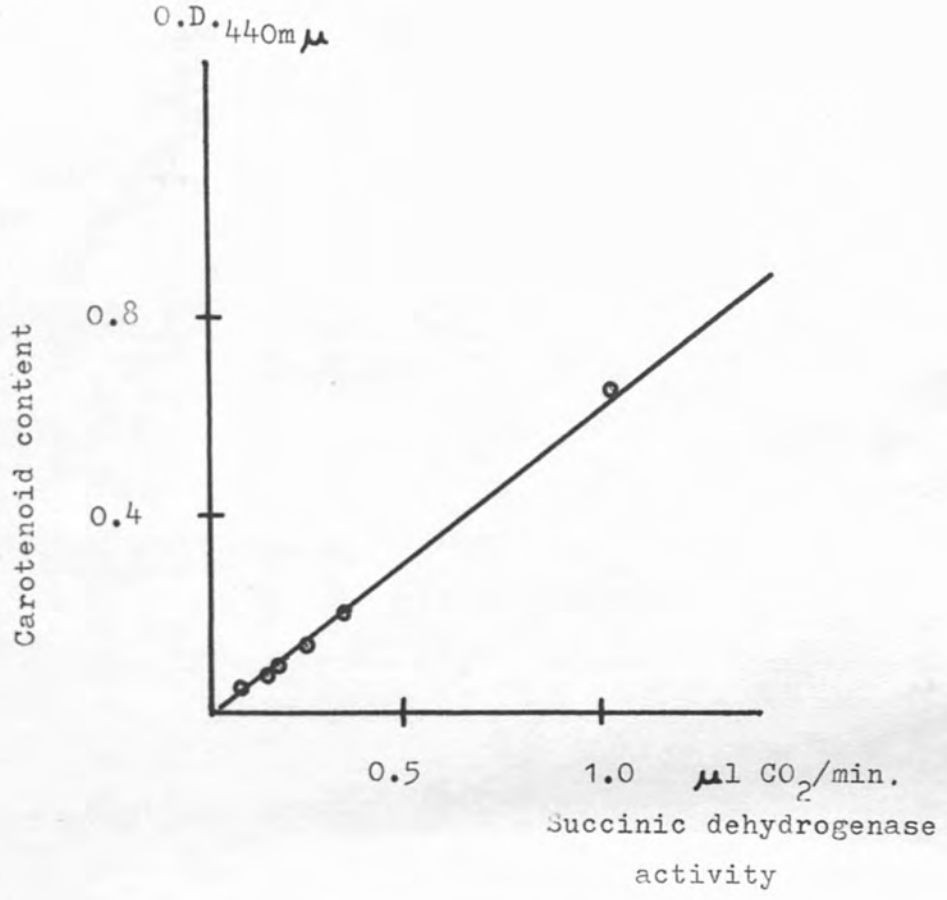


Figure 13. Comparison of the carotenoid content and succinic dehydrogenase activity of fractions of a cauliflower bud homogenate separated on a gradient composed of 1.25, 1.35, and 1.45M sucrose. (see table 14)

Table 14. Analysis of fractions of a cauliflower bud homogenate separated on a density gradient composed of 1.25, 1.35, and 1.45M sucrose.

For details of homogenisation procedure, differential and gradient centrifugation, nomenclature of fractions, division and washing of pellets, and the assay of carotenoid content and succinic dehydrogenase activity, see page 31 to 34.

Table 14.

	Homogenate sample	Pellet 1	Pellet X	Pellet 1.25	Pellet 1.35	Pellet 1.45
Carotenoid content ($O.D._{440m\mu}$)						
Estimated min. value	0.118	0.63	0.202	0.054	0.065	0.100
Estimated max. value	0.127	0.66	0.228	0.063	0.075	0.110
Average value	0.122	0.64	0.218	0.058	0.070	0.105
Succinic dehydrogenase activity ($\mu l.CO_2$/min.)	0.252	1.03	0.35	0.075	0.146	0.168
Dry weight (g.)	0.044	0.261	0.057	0.023	0.037	0.026
Average Carotenoid/ g. dry weight	2.6	2.4	3.8	2.6	1.9	4.0
Succinic dehydrogenase activity/g. dry weight	5.8	3.9	6.1	3.3	4.0	6.5
Ratio $\frac{\text{Average Carotenoid content}}{\text{Succinic dehydrogenase activity}}$	0.49	0.60	0.62	0.71	0.47	0.62

Mean ratio $\frac{\text{Average Carotenoid content}}{\text{Succinic dehydrogenase activity}}$ 0.585

Standard deviation 15.4%

Percentage recoveries:-

Carotenoid	90%
Succinic dehydrogenase activity	78%
Dry weight	102%

and dry weight varied between 80-94%, 73-100% and 64-105% respectively. This loss was thought to be sufficiently small to represent experimental error rather than carotenoid oxidation or inactivation of succinic dehydrogenase. Therefore, it was concluded that the sedimentation patterns of particles containing carotenoid and succinic dehydrogenase activity were similar.

Fractionation by both differential centrifugation and discontinuous sucrose density gradient centrifugation showed that the distribution patterns of carotenoid and succinic dehydrogenase activity were closely associated and that particles containing these were virtually inseparable. If one deduces from this that carotenoids were present in mitochondria in the homogenate, then the conclusion is substantiated by the observations that changes in the distribution of succinic dehydrogenase activity on the sucrose gradients, which appeared to be caused by the state of the cauliflowers and the sharpness of the blades amongst other things affected the distribution of carotenoids in the same manner.

4. Density of Mitochondria

The results of the discontinuous gradient experiments illustrated that carotenoids and succinic dehydrogenase, the mitochondrial marker, were present in particles with a wide range of densities. One can combine these results to determine the approximate density distribution pattern of the particles.

The method used for the assay of succinic dehydrogenase activity was not very sensitive and therefore in certain fractions activity was not detected. However, carotenoid-

containing particles were shown to follow the same sedimentation patterns as mitochondria and the method for assaying carotenoid was more sensitive for smaller amounts of material. It was therefore possible to ascertain the density distribution curve of mitochondria from the results of the carotenoid analyses.

The data from tables 11, 12 and 13 can be summarised by superimposing the histograms firstly of the carotenoid content (figure 14a), secondly for dry weight measured as a percentage of the dry weight recovered from the gradient (figure 15a), and thirdly for carotenoid content per g. dry weight of the centrifugal fractions in each experiment (figure 16a). These results can be interpreted to give a qualitative assessment of the distribution patterns of mitochondria (figure 14b), dry weight (figure 15b), and the degree of contamination of the mitochondria with other subcellular material (figure 16b). It would be extremely difficult to make a quantitative assessment as each horizontal line represents the total value for all the material with a density represented by its span and the patterns were slightly affected by the variation in sedimentation behaviour in different experiments.

The majority of mitochondria were located between 1.25 and 1.35M sucrose (i.e. had densities between 1.165 and 1.179) and few were found between 1.1 and 1.15M sucrose (i.e. few had densities of 1.145 to 1.151) (figure 14); the majority of material on the gradient was between 1.3 and 1.4M sucrose (i.e. had a density of 1.172 to 1.185) (figure 15); and mitochondria located between 1.25 and 1.3M sucrose and 1.1 and 1.15M sucrose (i.e. having densities between 1.165 and 1.172 and also 1.145 and 1.151) were least contaminated with other subcellular material (figure 16).

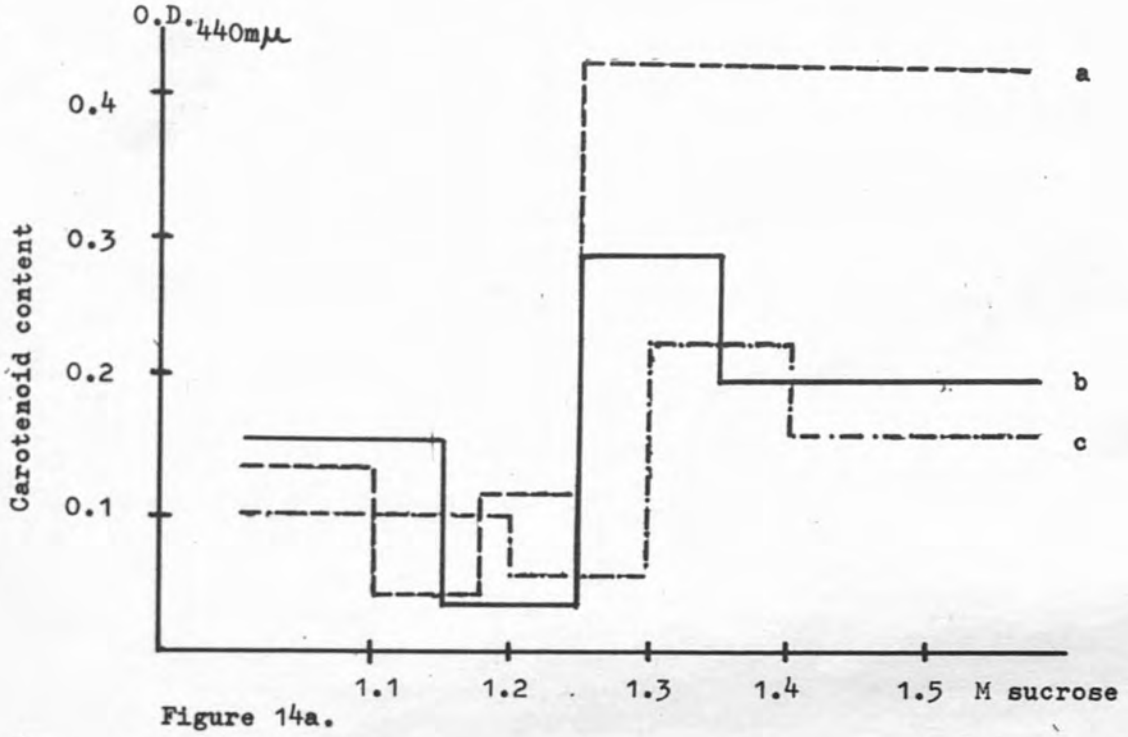


Figure 14a.

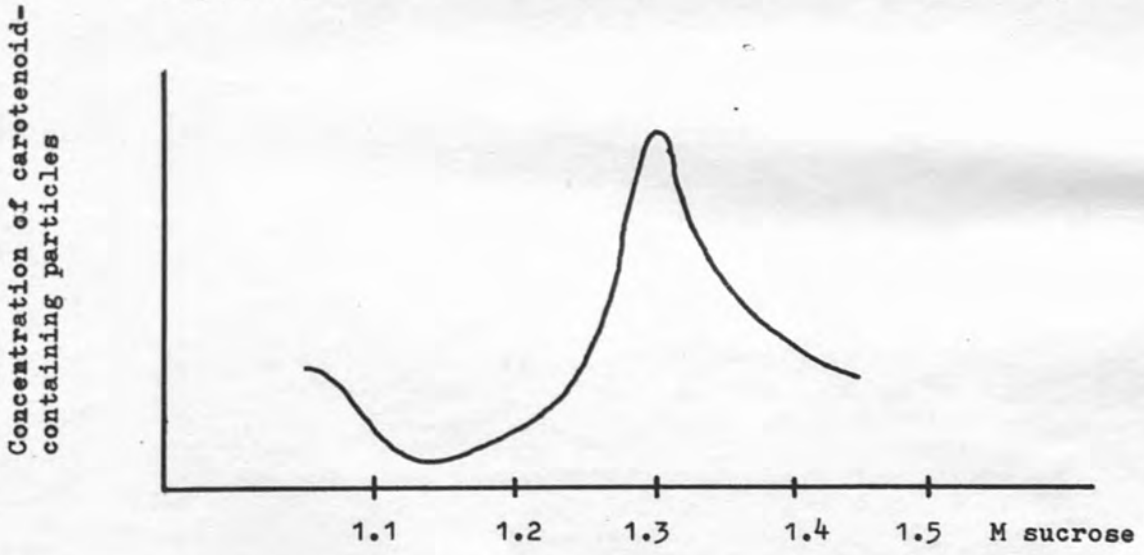


Figure 14b.

Figure 14a. Profiles of histograms of the carotenoid content of fractions separated on density gradients. The full results of experiments a,b,and c are shown in tables 11,12,and 13 respectively.

Figure 14b. The combined effect when the profiles in figure 14a. are superimposed. This shows the concentration of carotenoid-containing particles at different densities.

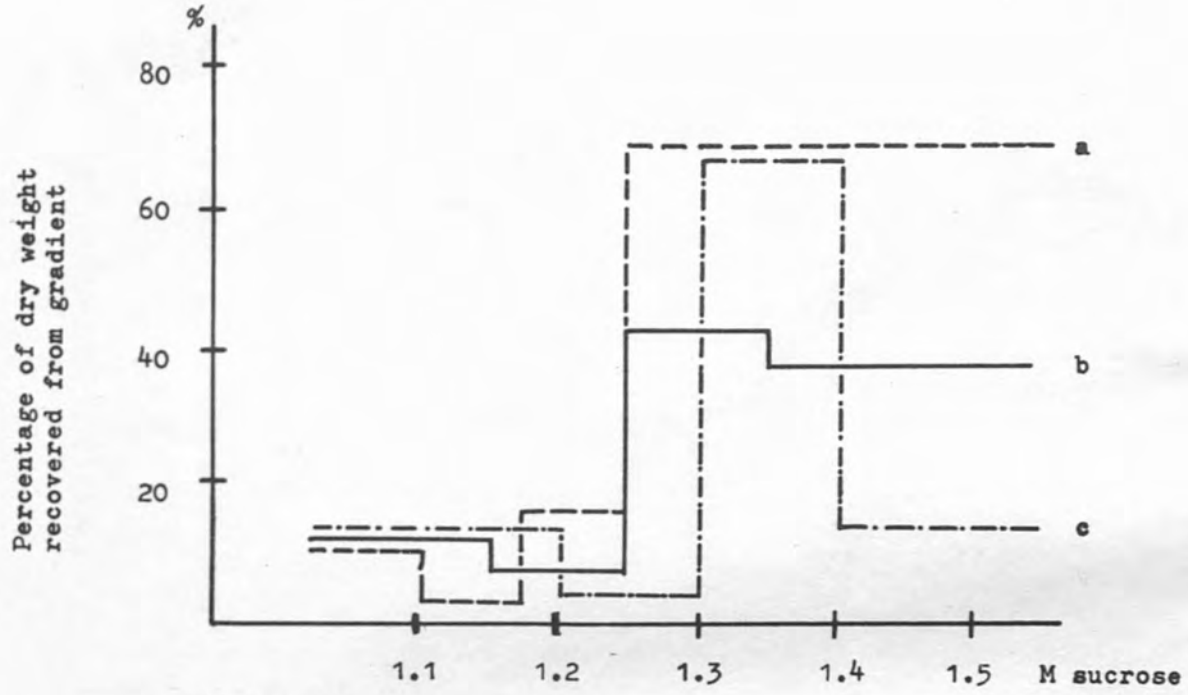


Figure 15a.

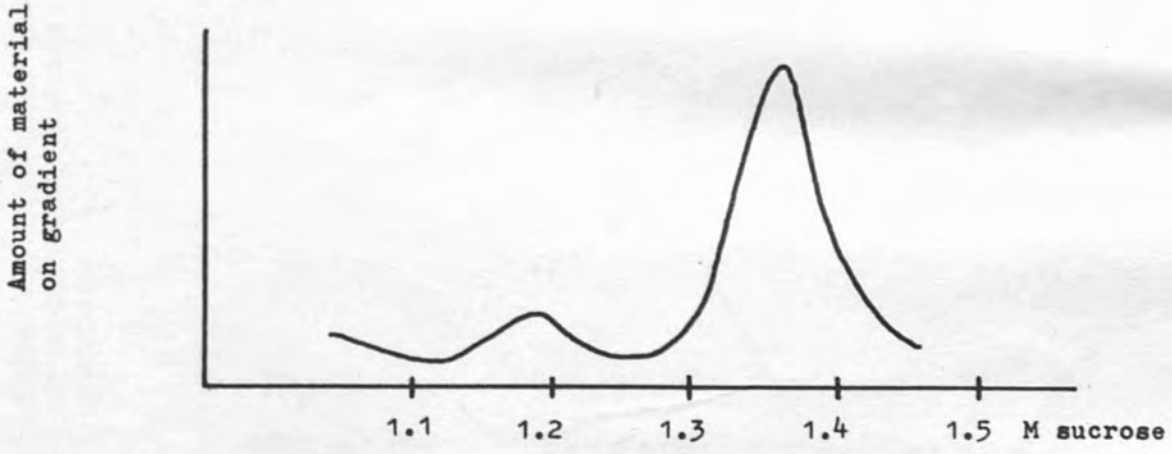


Figure 15b.

Figure 15a. Profiles of histograms of the percentage of material recovered from the gradient in the fractions. The full results of experiments a,b, and c are shown in tables 11,12, and 13 respectively.

Figure 15b. The combined effect when the profiles in figure 15a. are superimposed. This shows the amount of material which has a density equivalent to that of the molarity of sucrose at any point on the gradient.

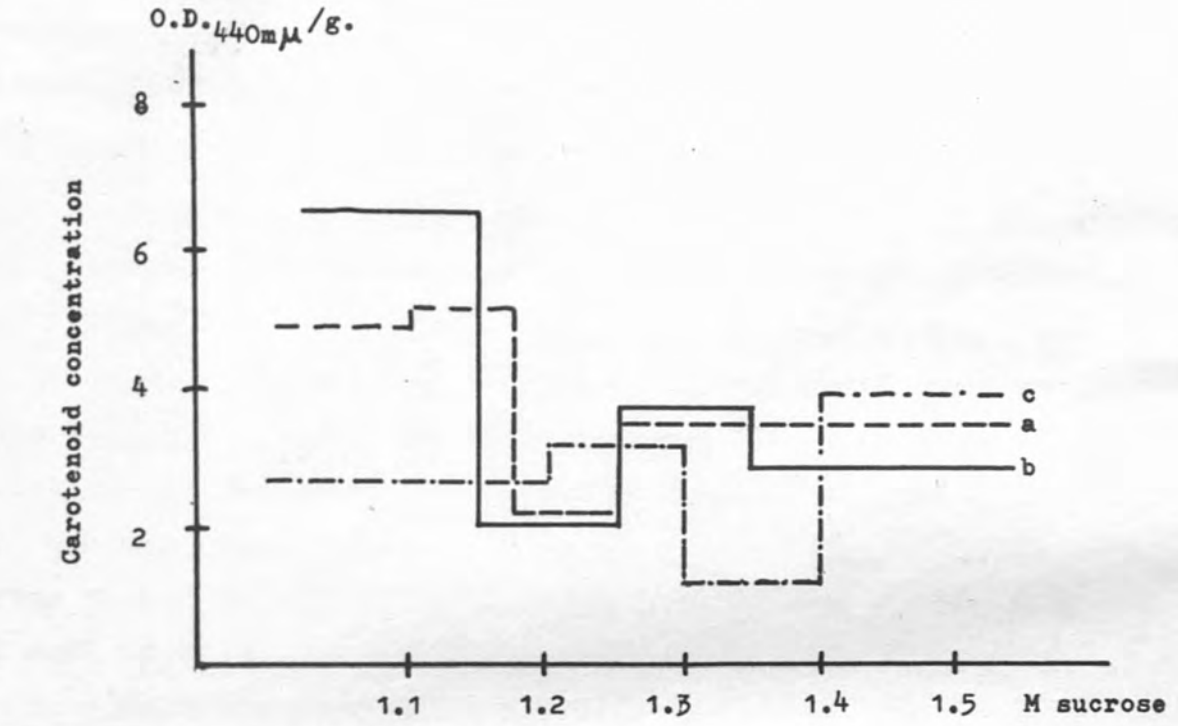


Figure 16a.

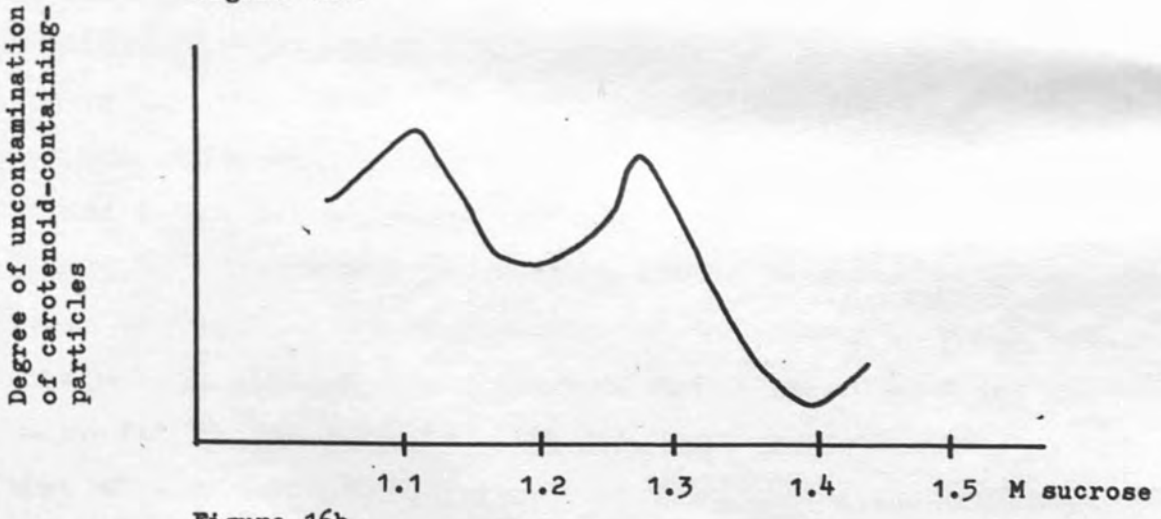


Figure 16b.

Figure 16a. Profiles of histograms of the carotenoid content of 1g. of the fractions recovered from the density gradients. a,b, and c are derived from results which appear in tables 11,12, and 13 respectively.

Figure 16b. The combined effect when the profiles in figure 16a. are superimposed. This illustrates the degree of uncontamination of the carotenoid-containing particles at various densities.

5. Supplementary Experiments

For the purpose of comparison two supplementary experiments were performed. In the first the sedimentation pattern of carotenoid - containing particles was compared to that of glucose-6-phosphatase activity, the latter being frequently associated with the microsomes. In the second, chlorophyll sedimentation was compared with that of succinic dehydrogenase activity in cauliflower stalks.

(a) To compare the sedimentation patterns of carotenoid and glucose-6-phosphatase activity in buds the homogenisation and differential centrifugation procedure outlined on pages 31 to 34 were followed. The centrifugal fractions were divided into two equal parts, one being assayed for glucose-6-phosphatase activity and the other for carotenoid following lyophilisation and weighing (see page 27). Both results were expressed in terms of dry weight, which was the same in the two samples of each fraction.

From the results in table 15 and figure 17 it can be seen that pellet 2 possessed the greatest carotenoid concentration but pellet 4 had the highest glucose-6-phosphatase activity in unit dry weight. Similarly the sequence of the fractions containing decreasing amounts of carotenoid was 2, 3, 1 and 4 but for glucose-6-phosphatase activity this was 1, 3, 4 and 2. Therefore there appeared to be no correlation between the sedimentation patterns of the carotenoid-containing particles and glucose-6-phosphatase activity. This was illustrated by the fact that the standard deviation of the ratios of the carotenoid content to glucose-6-phosphatase activity was 70%.

(b) To compare the sedimentation patterns of mitochondria and chlorophyll-containing plastids of cauliflower stalk tissue,

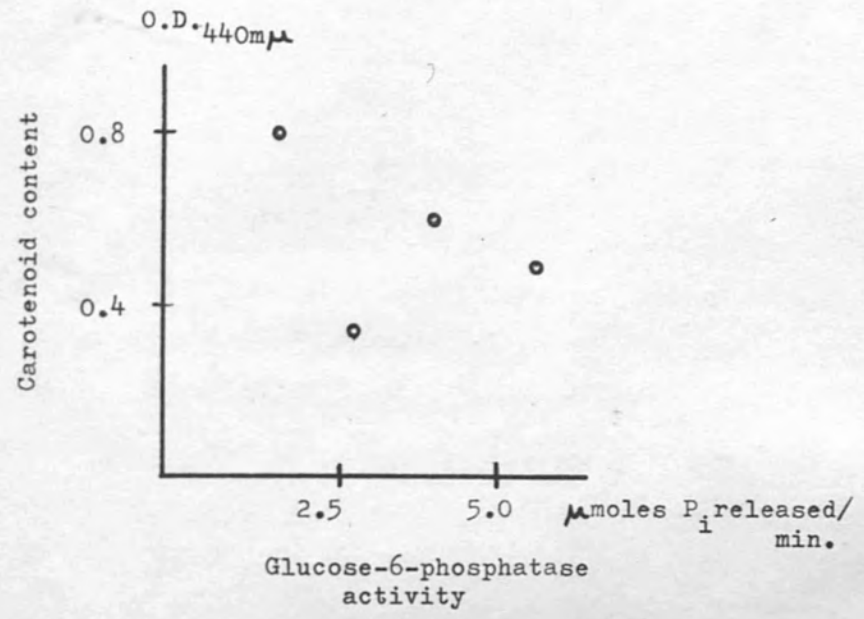


Figure 17. Comparison of the carotenoid content and glucose-6-phosphatase activity of fractions of a cauliflower bud homogenate separated by differential centrifugation. (see table 15)

Table 15. Analysis of the carotenoid content and glucose-6-phosphatase activity of fractions of a cauliflower bud homogenate separated by differential centrifugation.

For details of homogenisation procedure, differential centrifugation, nomenclature of fractions, division and washing of pellets, and the methods for the assay of carotenoid content and glucose-6-phosphatase activity, see pages 31, 32, and 26.

Table 15.

	Pellet 1	Pellet 2	Pellet 3	Pellet 4
Carotenoid content ($O.D._{440m\mu}$)	0.565	0.80	0.62	0.345
Glucose-6-phosphatase activity (μ moles P_i released/min.)	5.5	1.88	4.05	2.7
Dry weight (g.)	0.159	0.058	0.110	0.046
Carotenoid/g. dry weight	3.56	13	5.6	7.5
Glucose-6-phosphatase activity/g. dry weight	34.6	32.4	36.8	58.8
Ratio $\frac{\text{Carotenoid content}}{\text{Glucose-6-phosphatase activity}}$	0.125	0.425	0.153	0.128
Mean ratio $\frac{\text{Carotenoid content}}{\text{Glucose-6-phosphatase activity}}$		0.145		
Standard Deviation		70%		

the homogenisation and differential centrifugal procedures outlined on pages 31 to 34 were followed. Chlorophyll was extracted from the fractions analysed by the method described on page 27 and expressed in terms of the optical density of the extract in 1ml for a 1cm light path. Both the chlorophyll content and succinic dehydrogenase activity of each fraction were expressed in terms of the dry weight of the chlorophyll sample.

The results of this experiment, given in table 16, show that although pellet 2 contained the largest amounts of chlorophyll and succinic dehydrogenase activity, these were present in the greatest concentrations in pellets 1 and 3 respectively. When the carotenoid content of the fractions was plotted against the succinic dehydrogenase activity no linear relationship was obtained (figure 18) and the standard deviation of the ratios of chlorophyll content and succinic dehydrogenase activity was 72.5%. Therefore there was no correlation between the sedimentation patterns of chlorophyll content and succinic dehydrogenase activity in particles from cauliflower stalks.

These two experiments therefore illustrated the results to be expected when a comparison is made between two substances which are located in different subcellular particles. The largest amounts and also the greatest concentrations may be present in different fractions, no linear relationship is observed when the amounts of these two substances are compared in each fraction, and a standard deviation of the ratio of these two substances is greater than can be attributed to experimental error.

Figure 18. Comparison of the chlorophyll content and succinic dehydrogenase activity of fractions of a cauliflower stalk homogenate separated by differential centrifugation. (see table 16)

Table 16. Analysis of the chlorophyll content and succinic dehydrogenase activity of fractions of a cauliflower stalk homogenate separated by differential centrifugation.

For details of homogenisation procedure, differential centrifugation, nomenclature of fractions, division and washing of pellets, and the assay of chlorophyll content and succinic dehydrogenase activity, see pages 31 to 34.

Table 16.

	Homogenate sample	Pellet 1	Pellet 2	Pellet 3	Pellet 4
Chlorophyll content ($O.D._{660m\mu}$)	0.45	1.28	1.66	0.328	0.134
Succinic dehydrogenase activity ($\mu l.CO_2$/min.)	0.124	0.153	0.51	0.179	0.085
Dry weight (g.)	0.168	0.382	0.633	0.128	0.14
Chlorophyll/g. dry weight	0.27	4.5	2.7	1.84	0.95
Succinic dehydrogenase activity/g. dry weight	0.74	0.40	0.8	1.4	0.6
Ratio $\frac{\text{Chlorophyll content}}{\text{Succinic dehydrogenase activity}}$	3.64	8.35	3.26	1.83	1.58

Mean ratio $\frac{\text{Chlorophyll content}}{\text{Succinic dehydrogenase activity}}$ = 3.73

Standard deviation = 72.5%

6. Electron Microscopy

Electron micrographs of fractions of a cauliflower bud homogenate, separated on a sucrose gradient, were obtained to exclude the possibility of the presence of a non-mitochondrial type of particle containing carotenoid which followed a similar sedimentation pattern to mitochondria, the distribution of which was affected in the same manner by factors producing changes in the sedimentation pattern of mitochondria.

The density gradient fractions were divided into two equal parts, one of which was washed as described on page 32, lyophilised, and assayed for carotenoid. The carotenoid content of the fractions is shown in table 17. The other half of each fraction was washed twice with the blending medium, sedimented at 38,000g for 30 minutes, and resuspended in a small volume of blending medium. Electron micrographs were taken of samples of these suspensions by the negative staining technique described by Cunningham and Crane (93), using 2% phosphotungstic acid.

Table 17. Carotenoid content of sucrose density gradient fractions examined by electron microscopy.

	Pellet 1.4	Pellet 1.3	Pellet 1.2	Pellet X
Total carotenoid ($O.D._{440m\mu}$)	0.045	0.202	0.017	0.084
Dry weight g.	0.0157	0.0468	0.0078	0.0174
Carotenoid content/ g. dry weight	2.86	4.32	2.2	4.8

Unfortunately, it was not possible to identify mitochondria by the criterion of Cunningham and Crane (93), who detected "knob structures" on the extruded surface of the inner mitochondrial

membrane. Certain particles which appeared to resemble mitochondria in shape were observed in pellet 1.3, however, this being the fraction containing the greatest amount of carotenoid. Similar particles were observed in pellets 1.4 and 1.2 but were less regular in appearance and the lightest fraction, pellet X, contained smaller membraneous particles, perhaps fragments of the particles in more dense fractions (see figure 19). The proplastids which Cunningham and Crane observed could not be distinguished.

The electron micrographs did not clearly show all that was hoped for and therefore it was not possible to draw definite conclusions concerning the presence or absence of a non-mitochondrial carotenoid-containing particle.

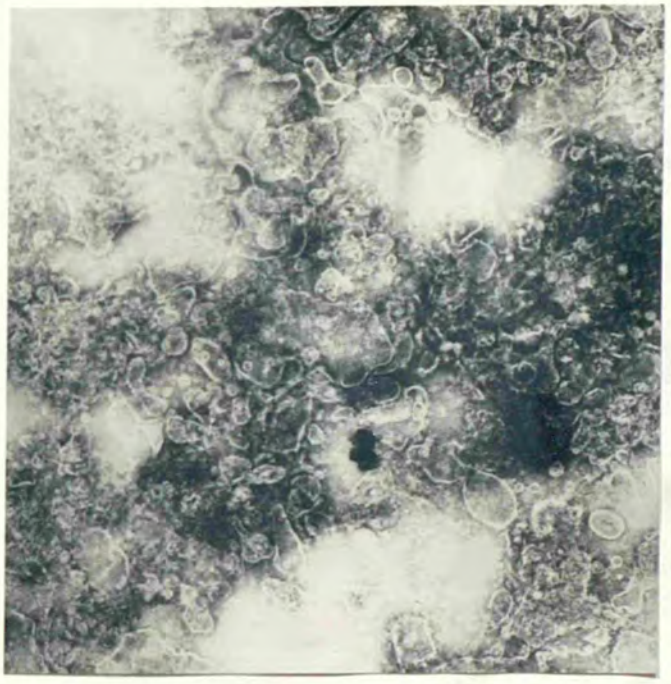
7. Summary

Analysis of acetone extracts of the whole cauliflower bud tissue showed the presence of 4 major fractions:- β-carotene (12%), lutein (33%), violaxanthin (44.5%), and neoxanthin (10.5%).

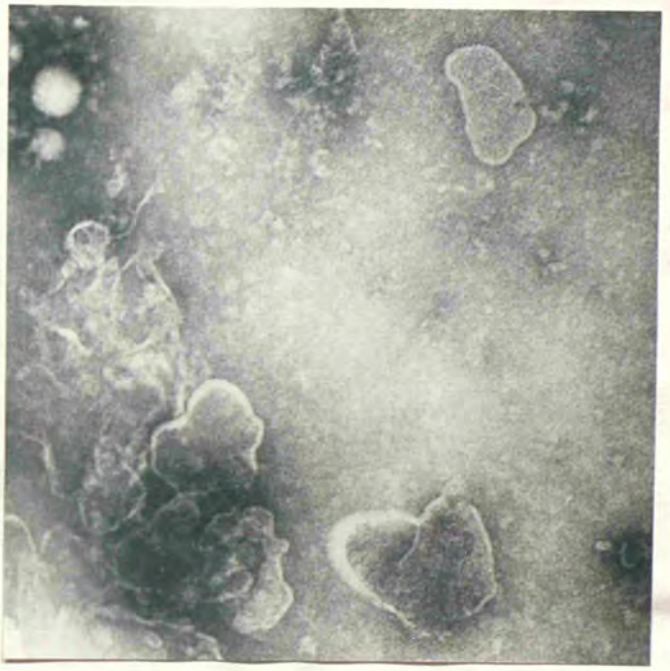
When the homogenised tissue was subjected to differential centrifugation or discontinuous sucrose density gradient centrifugation, a direct correlation was invariably observed between the distribution of carotenoid and succinic dehydrogenase activity, the mitochondrial marker, in the fractions. Supplementary experiments demonstrated that a difference in sedimentation patterns was readily distinguished when the distribution patterns of two substances located in different subcellular particles were compared.

The majority of carotenoid-containing particles were shown to have densities equivalent to approximately 1.3M sucrose although the pigment was distributed in particles with densities equivalent to 1.0 to 1.6M sucrose.

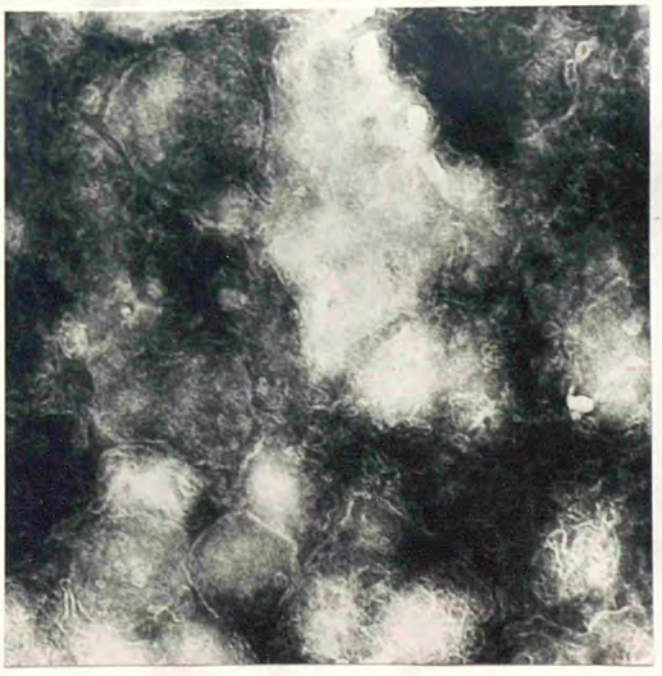
Figure 19. Electron micrographs of centrifugal fractions of a cauliflower bud homogenate fractionated on a density gradient composed of 1.2, 1.3, and 1.4M sucrose.



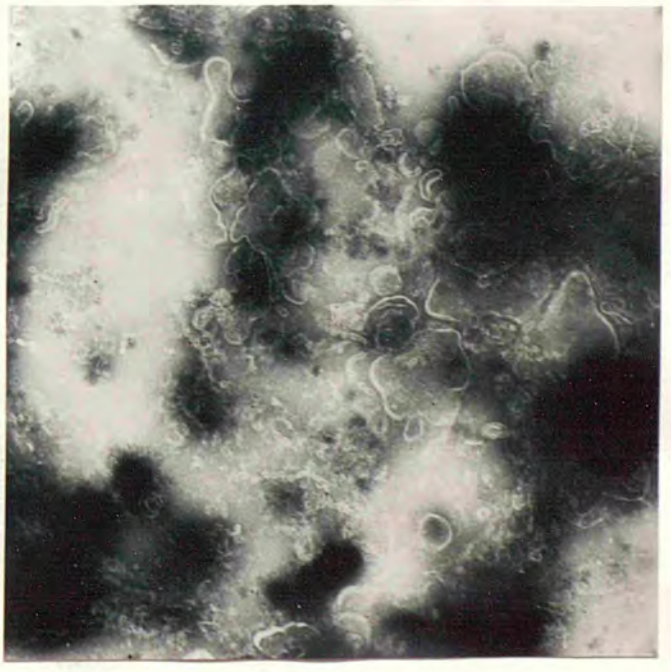
Pellet X



Pellet 1.2



Pellet 1.3



Pellet 1.4

Electron micrographs failed to distinguish the presence of a non-mitochondrial particle sedimenting in the same way as mitochondria which may have contained carotenoids.

Chapter 4.

The Presence of Carotenoids in Heart Muscle Mitochondria.

1. Introduction

The presence of carotenoid in heart muscle mitochondria is suggested by the results of a number of workers (61, 62, 63, 64, 65, 75) but none of their methods has excluded the possibility of contamination of mitochondria by carotenoids originating from other sources, such as blood or lipid stores.

A method of investigation, similar to that applied in the previous chapter, was initiated in an attempt to demonstrate that carotenoids were associated with the mitochondrial fraction of ox heart muscle homogenates and could not be separated from them by centrifugation. In initial experiments, on hearts transported from the slaughter house at ambient temperature, a wide divergence was observed in the sedimentation patterns of succinic dehydrogenase and carotenoid. Potassium cyanide was therefore incorporated in the washing and blending media of the following experiment in an attempt to inhibit any haemoglobin - catalysed carotenoid breakdown, thought to occur following haemolysis of the red corpuscles. This procedure, however, produced an even greater difference in the sedimentation patterns of carotenoid and succinic dehydrogenase activity.

To minimise carotenoid oxidation during transport, hearts were packed in ice and water and attempts were made to remove selectively extramitochondrial carotenoids, thought to be present in the blood, by incubation of the minced muscle with haemolysed blood. Verification that carotenoid was oxidised during incubation with haemolysed blood was then obtained by an examination of heart muscle and blood carotenoids before

and after incubation following haemolysis.

Attempts were also made to extract carotenoid from pig heart muscle but these proved unsuccessful.

2. Hearts Transported at Ambient Temperature

(a) Initial experiments were designed to examine the sedimentation patterns of carotenoid-containing particles and mitochondria. The results of a typical fractionation experiment, following the procedures outlined in pages 41 to 43, appear in table 18. From this it can be seen that carotenoid and succinic dehydrogenase, the mitochondrial marker, were distributed throughout all the centrifugal fractions. However, pellets 1 and 2 contained the largest amounts of carotenoid and succinic dehydrogenase respectively while they were present in the greatest concentration in pellets 2 and 3 respectively. Thus, no correlation was observed between the sedimentation patterns of carotenoid-containing particles and succinic dehydrogenase activity. This difference is illustrated by the fact that the standard deviation of the ratios of carotenoid content to succinic dehydrogenase activity of the fractions was 43.6%.

(b) It was thought that carotenoid was being lost by haemoglobin-catalysed oxidations (94) which may have selectively reduced the carotenoid content of certain fractions. This oxidation can be inhibited by 0.198M KCN (94) which was therefore incorporated into the washing and blending media (see page 42) used in the preparation of centrifugal fractions in the following type of experiment.

The results of a typical experiment appear in table 19. These resemble the results of the previous experiment in as much as they illustrate that carotenoid-containing particles sedimented in a different manner from mitochondria. The

Table 18. Comparison of the sedimentation patterns of carotenoid-containing particles and mitochondria of ox heart muscle which had been transported from the slaughter house at ambient temperature.

	Pellet 1	Pellet 2	Pellet 3	Pellet 4	Pellet 5	Supernatant
Carotenoid content (μg.)	0.340	0.605	0.146	0.054	0.019	0.135
Succinic dehydrogenase activity (μl O_2/ min.)	3.43	3.15	1.55	0.325	0.0295	1.46
Protein content (mg.)	7.8	2.1	0.76	0.26	0.9	3.6
Carotenoid concentration (μg./mg. protein)	0.042	0.328	0.177	0.207	0.216	0.039
Succinic dehydrogenase activity/mg. protein	0.33	1.50	2.05	1.25	0.033	0.41
Ratio $\frac{\text{Carotenoid content}}{\text{Succinic dehydrogenase activity}}$	0.10	0.19	0.09	0.18	0.07	0.09
Mean ratio $\frac{\text{Carotenoid content}}{\text{Succinic dehydrogenase activity}}$		0.120				
Standard deviation		43.6%				

Experimental details of washing procedure, homogenisation, centrifugal fractionation, nomenclature of pellets, and the assay of carotenoid and succinic dehydrogenase activity appear on pages 41 to 43.

Table 19. Comparison of the carotenoid content and succinic dehydrogenase activity of centrifugal fractions of ox heart muscle, transported from the slaughter house at ambient temperature and separated in 0.195M KCN.

	Pellet 1	Pellet 2	Pellet 3	Pellet 4	Pellet 5	Supernatant
Carotenoid content (μg.)	4.8	1.4	0.32	0.31	0.11	0.46
Succinic dehydrogenase activity ($\mu l O_2$/min.)	0.92	2.64	0.62	1.46	0.29	1.17
Protein content (mg.)	4.6	2.9	0.75	0.93	0.18	6.5
Carotenoid concentration (μg./mg. protein)	0.10	0.49	0.43	0.33	0.65	0.069
Succinic dehydrogenase activity/mg. protein	0.02	0.91	0.83	1.57	1.64	0.18
Ratio $\frac{\text{Carotenoid content}}{\text{Succinic dehydrogenase activity}}$	5.2	0.54	0.54	0.23	0.39	0.27
Mean ratio $\frac{\text{Carotenoid content}}{\text{Succinic dehydrogenase activity}}$		1.19				
Standard deviation		165%				

Experimental details of the washing procedure, homogenisation, centrifugal fractionation, nomenclature of fractions, and the methods for the assay of carotenoid content and succinic dehydrogenase activity appear on pages 41 to 43.

largest amounts of carotenoid and succinic dehydrogenase activity were present in pellets 1 and 2 respectively while the greatest concentration of both was found in pellet 5. However, there appeared to be no correlation between the sedimentation patterns of particles containing carotenoid and succinic dehydrogenase activity, which was illustrated by a standard deviation in the ratios of 165%.

The mean ratio of 1.19 was greater than that of 0.12 observed in the previous type of experiment, indicating that carotenoid degradation was inhibited by KCN. This was thought to imply that carotenoid had probably been degraded in the previous experiment by contact with haemoglobin in the blood.

3. Hearts Transported at 0° C.

(a) On the assumption that haemoglobin-catalysed carotenoid oxidation occurred, attempts were made to minimise the loss of carotenoid by surrounding the hearts in ice and water immediately following removal from the ox.

The first experiment on heart muscle, described on page 88, was repeated on muscle transported at 0° C and the results shown in table 20 were obtained. No correlation was observed between the sedimentation patterns of particles containing carotenoid and succinic dehydrogenase activity. Although pellet 2 contained the largest amounts of carotenoid and succinic dehydrogenase activity, these were present in greatest concentrations in different fractions and the standard deviation of the ratio of these perameters increased to 172%. The increase in the mean ratio and standard deviation illustrated that carotenoid may have previously been lost during transport of the hearts at ambient temperature and the conclusion that extramitochondrial carotenoid was present was substantiated by an increase in the standard deviation of the ratios.

Table 20. Comparison of the carotenoid and mitochondrial contents of centrifugal fractions of ox heart muscle transported from the slaughter house at 0° C.

	Pellet 1	Pellet 2	Pellet 3	Pellet 4	Pellet 5	Supernatant	Homogenate
Carotenoid content (μg.)	7.6	13.9	1.15	1.46	1.19	5.9	2.16
Succinic dehydrogenase activity (μl CO_2/min.)	80	238	43	46	1.9	2.7	16
Protein content (mg.)	108	42	19.6	6.9	2.55	37	10.5
Carotenoid concentration (μg./mg. protein)	0.07	0.33	0.06	0.21	0.47	1.6	0.26
Succinic dehydrogenase activity / mg. protein	0.74	2.64	2.19	6.7	0.75	0.07	1.55
Ratio $\frac{\text{Carotenoid content}}{\text{Succinic dehydrogenase activity}}$	0.094	0.058	0.027	0.034	0.63	2.18	0.135

Mean ratio $\frac{\text{Carotenoid content}}{\text{Succinic dehydrogenase activity}}$ 0.452 Standard deviation 172%

Percentage recoveries:- Carotenoid 73% Succinic dehydrogenase activity 108% Protein 102%

For experimental details of washing procedures, homogenisation, centrifugal fractionation, nomenclature of fractions, and the methods for the assay of carotenoid content and succinic dehydrogenase activity, see pages 41 to 43.

(b) Blood carotenoids are attached to the globulin fraction (95) and should theoretically be removed by water washing. The possibility exists, however, that if the carotenoid were not tightly attached it could be released during washing and become associated with suitable lipid membranes. A less likely alternative is that the carotenoid-carrying globulin could become attached to the membranes. Assuming either of these possibilities, an attempt was made to remove selectively blood carotenoids by suspending the minced muscle in a small volume of ice and water preceding homogenisation. It was envisaged that this would result in haemolysis of the erythrocytes exposing blood carotenoids to haemoglobin-catalysed degradation. Subsequent washings would remove haemoglobin, which would not then be available to attack carotenoids of subcellular particles of heart muscle cells exposed during later homogenisation.

Two experiments were performed in which the mince was incubated with a small volume of ice and water for one hour and two hours respectively, washed free of haemoglobin, and fractionated. The results of these experiments appear in tables 21 and 22 respectively.

A decrease in the mean ratio of the carotenoid content to succinic dehydrogenase activity occurred, suggesting a loss of carotenoid, but the standard deviation of the ratios also decreased. In the second experiment pellet 5 contained both the largest amount of pigment and enzyme activity, these being most concentrated in the second pellet, and the standard deviation was within the probable limits of experimental error to be expected if carotenoids were associated only with the mitochondrial fraction.

Therefore, after two hours incubation of the ox heart muscle mince with a small volume of ice and water, followed by washing, the sedimentation patterns of carotenoid and succinic dehydrogenase activity were similar.

Table 21. Comparison of the carotenoid and mitochondrial contents of centrifugal fractions of ox heart mitochondria following partial oxidation of carotenoids by preincubation for one hour with haemolysed blood.

	Pellet 1	Pellet 2	Pellet 3	Pellet 4	Pellet 5	Supernatant	Homogenate
Carotenoid content (μg.)	44.9	9.2	9.6	2.6	1.2	1.9	7.9
Succinic dehydrogenase activity (μl CO_2/min.)	121	64.5	41.7	24.8	1.82	2.71	37.8
Protein content (mg.)	262	30.8	22.5	15.0	3.12	60.0	58.0
Carotenoid concentration (μg./mg. protein)	0.17	0.24	0.43	0.13	0.43	0.031	0.134
Succinic dehydrogenase activity /mg. protein	0.47	2.10	1.84	1.65	0.58	0.05	0.65
Ratio $\frac{\text{Carotenoid content}}{\text{Succinic dehydrogenase activity}}$	0.370	0.142	0.230	0.088	0.679	0.679	0.208

Mean ratio $\frac{\text{Carotenoid content}}{\text{Succinic dehydrogenase activity}}$ 0.308 Standard deviation 71.5%

Percentage recoveries:- Carotenoid 114% Succinic dehydrogenase activity 75% Protein 73%

For experimental details of incubation, washing, homogenisation, centrifugal fractionation, nomenclature of pellets, and assay methods for carotenoid and succinic dehydrogenase, see pages 41 to 43.

Table 22. Comparison of the carotenoid and mitochondrial contents of centrifugal fractions of ox heart muscle following partial oxidation of carotenoids by preincubation with haemolysed blood for two hours.

	Pellet 1	Pellet 2	Pellet 3	Pellet 4	Pellet 5	Supernatant	Homogenate
Carotenoid content (μg.)	25.9	8.5	4.6	2.4	0.73	3.6	7.7
Succinic dehydrogenase activity (μl CO_2/min.)	88.0	31.0	14.8	8.1	2.3	12.3	29.0
Protein (mg.)	468	18.9	10.0	6.3	1.95	34.4	67.5
Carotenoid concentration (μg. / mg. protein)	0.054	0.45	0.44	0.39	0.37	0.10	0.12
Succinic dehydrogenase activity / mg. protein	0.19	1.64	1.48	1.28	1.16	0.36	0.43
Ratio $\frac{\text{Carotenoid content}}{\text{Succinic dehydrogenase activity}}$	0.287	0.273	0.300	0.300	0.319	0.292	0.271

Mean ratio $\frac{\text{Carotenoid content}}{\text{Succinic dehydrogenase activity}}$ 0.292 Standard deviation 5.8%

Percentage recoveries:- Carotenoid 90% Succinic dehydrogenase activity 82% Protein 127%

For experimental details of incubation, washing, homogenisation, centrifugal fractionation, nomenclature of pellets, and assay methods for carotenoid and succinic dehydrogenase activity, see pages 41 to 43.

Percentage recoveries from the two initial experiments were not estimated due to an oversight. However, it was thought that in the subsequent experiments the recovery of carotenoid, succinic dehydrogenase activity and protein, following centrifugation, was satisfactory. The loss or gain of each substance was thought to be a reflection of the limitations of the method and also experimental error rather than possessing any physiological significance. For example, errors occurring during the pipetting of samples for the assay of succinic dehydrogenase activity and protein caused by clumping and sedimentation of particles may have been responsible for high estimated recoveries of succinic dehydrogenase activity and protein.

It may therefore be concluded that carotenoid was associated with the mitochondrial fraction and was also present in an extramitochondrial fraction, possibly the blood. This second type of carotenoid was removed by incubation with haemolysed blood preceding homogenisation of the muscle.

4. Analysis of Blood and Heart Carotenoids

To verify the latter conclusion of the preceding paragraph, the carotenoid content of blood and ox heart muscle was examined following a number of different treatments and the extracted carotenoids were analysed on alumina columns (Brockmann activity 1.). The results of these extractions, outlined below, appear in table 23.

i. Blood was haemolysed at the slaughter house and carotenoids extracted immediately by the addition of two volumes of alkaline methanol (see page 30).

ii. Haemolysed blood was incubated for two hours at ambient temperature and carotenoids were then extracted.

A decrease of 55% in extracted carotenoids resulted from incubation of the haemolysed blood.

iii. Carotenoids were extracted from beef heart muscle which was lyophilised immediately after mincing (see page 30). The extinction of this extract therefore represented the total carotenoid extracted from the muscle and associated blood.

iv. Carotenoids were extracted from lyophilised minced heart muscle which had previously been washed to remove all haemoglobin (see page 41). More carotenoid per g. dry weight was extracted than following the treatment discussed in iii. This suggests firstly that carotenoids may have been more readily extracted following washing and secondly that blood carotenoids may not have been removed by washing.

v. Minced heart muscle was incubated for one hour at 0° C with a small volume of ice and water (see page 41), washed, and carotenoids were extracted from the lyophilised tissue. Carotenoid extracted / g. dry weight was lower than in the two preceding extractions.

vi. This treatment was similar to that in v. but the incubation period was increased to two hours. The carotenoid extracted was lower than in the preceding extractions suggesting that more carotenoid was lost during a longer period of incubation with the haemolysed blood.

The constituent carotenoids of the extracts were examined and three pigments were shown to be present, α - and β-carotene and a xanthophyll. The latter, representing a small percentage of the total carotenoid in each extract, was contaminated with a high u.v. absorbing material and it

was difficult therefore to estimate accurately. The percentages of the carotenes in the extract are shown in table 23.

Table 23. Analysis of carotenoids of blood and minced ox heart muscle.

Experiment no.	i	ii	iii	iv	v	vi
Tissue	Blood	Blood	Heart Muscle	Heart Muscle	Heart Muscle	Heart Muscle
Carotenoid content (mg./g. D.W.)			0.119	0.140	0.107	0.096
Carotenoid content (µg./ml. blood)	0.67	0.030				
Carotenoid analysis (excluding xanthophylls)						
% α-carotene	9.5		8.6	6.4	5.0	12.6
% β-carotene	90.5		91.5	93.6	95	87.4

For experimental details see text. α- and β-carotenes are expressed as a percentage of the carotenes recovered from the columns.

The results of the total carotenoid analysis of blood and heart muscle following various forms of pretreatment therefore supported the conclusion of section 3, that the carotenoids of blood and heart muscle (containing blood) decreased during incubation with haemolysed blood.

There is a trend in the results for the percentage of α- to β-carotene to decrease with decreasing blood levels except in the final estimation, extraction no. vi, which was performed on a different day from the others. The results

could therefore represent a difference in the carotenoid composition of blood and heart muscle tissue, or they may reflect experimental error.

5. Pig Heart Muscle

A number of methods were used in an attempt to extract carotenoid from pig heart muscle. These included extraction in solvent mixtures such as isopropanol/methanol and chloroform/methanol and extraction by ethanol and ether of ammonium sulphate fractions. These fractions were obtained from the supernatant of a homogenate, solublised in a solution of 0.05M Tris (pH 7.2) and 1% Tween 20 (96), which had previously been sonically disintegrated. KCN was not present in the blending medium, however, and therefore haemoglobin-catalysed carotenoid oxidation may have occurred. None of these methods was successful in demonstrating the presence of carotenoid in pig heart muscle.

6. Summary

Two types of carotenoid appeared to be present in ox heart muscle tissue; one was susceptible to the degrading effect of a blood constituent during incubation of the unhomogenised minced muscle, and was thought to originate from the blood. In the absence of this first type of carotenoid a second was seen to be associated with the mitochondrial fraction and could not be separated from it by differential centrifugation.

Chapter 5.

The Presence of Carotenoids in Mitochondria of the Yeast Rhodotorula rubra

1. Introduction

The subcellular location of carotenoids was examined in cultures of the red yeast Rhodotorula rubra. As in the two preceding series of experiments, carotenoid was shown to be present in all the centrifugal fractions obtained from the tissue examined, and therefore a similar line of investigation was employed in which the sedimentation patterns of carotenoid-containing particles and mitochondria were observed.

2. Growth of Cells in a Shaking Water Bath and Fermenter

Growth of cultures in a shaking water bath occurred in a relatively anaerobic atmosphere. A storage product was formed, which appeared during centrifugation as a white pellet, and this resulted in a high endogenous activity affecting the measurement of succinoxidase activity.

Conversely, an aerobic atmosphere existed when cells were grown in the fermenter. Excess lipid was formed which developed into a cream during centrifugation and carried dissolved carotenoid.

Both culture methods were therefore abandoned in favour of growth on agar plates, which did not have the disadvantages discussed above.

3. Growth on Agar Plates

The methods for the harvesting and breakdown of cells, centrifugal fractionation, and analysis of carotenoid content,

protein, and succinoxidase activity described on pages 43 to 45 were followed. Table 24 contains the succinoxidase activity of the centrifugal fractions obtained in this manner.

Table 24. Succinoxidase activity of the centrifugal fractions of Rhodotorula rubra.

Pellet	1	2	3	4	5
Succinoxidase activity (μl O_2/ hr.)	0	28.1	45	51.5	43

For experimental details and nomenclature, see pages 43 to 45.

The assay of carotenoid was affected by the presence of a contaminant which was extracted simultaneously. This contaminant persisted in both hypophasic and epiphasic carotenoid fractions and therefore attempts were made to purify the extracts by selective elution of carotenoids from magnesium oxide / hiflo supercel columns (97). Unfortunately, repeated use of the French press broke only a small percentage of the cells so that insufficient carotenoid was available for quantitative results to be obtained from these columns. From visual observation it was clear that the greatest carotenoid concentration was present in the 2nd pellet, the 4th and 5th containing the least. However, the greatest amount of succinoxidase activity appeared in the 4th fraction and the least in the 2nd.

The sedimentation patterns of carotenoid and succinoxidase activity were therefore dissimilar, from which it was concluded that carotenoid was present in some other particle other than, or as well as, the mitochondrion of the yeast Rhodotorula rubra.

Chapter 6.

Illumination of Cauliflower Bud Mitochondria

1. Introduction

From preceding chapters one can tentatively conclude that carotenoids exist in mitochondria isolated from cauliflower buds and ox heart. Many postulated roles for carotenoids relate to the effect of light on biological systems (see chapter 1) and it was therefore decided to examine the effect of light on mitochondrial respiration.

Cauliflower bud tissue mitochondria were selected for examination because considerable information on their isolation and properties had already been obtained. The rate of respiration of mitochondria prepared by the method described on page 45 was increased by 24% in the presence of 1×10^{-4}M dinitrophenol, suggesting that a degree of coupling existed.

Following examination of the effect of illumination on respiration, attempts were made to determine whether exogenous carotenoid influenced this light effect.

2. Effect of Light on Respiration and the Influence of Pretreatments

(a) The activity of succinoxidase, chosen as a convenient enzyme system by which to assay the respiratory chain, was measured manometrically by the method described on page 45.

In initial experiments in this series the rate of succinoxidase activity of a mitochondrial preparation was assayed in darkened and illuminated Warburg flasks immediately following centrifugal fractionation. Invariably, the rate of oxidation of the illuminated mitochondria fell below that of

those maintained in the dark after approximately 60 mins. An example of this effect is illustrated in figure 20a where the mitochondria were protected against a light-catalysed reduction in succinoxidase activity for approximately 60 mins.

(b) The centrifugal supernatant of a cauliflower bud homogenate has been shown to contain lipoxidase (see page 35). Incubation of the mitochondrial preparation with the supernatant would therefore reduce the carotenoid level. In the experiment whose results appear in figure 20, the influence on the light effect of preincubation of the mitochondria with the centrifugal supernatant for 2 hours at 0° C was compared with the basic light effect. Whereas protection existed for 60 mins. in the mitochondria which had not been preincubated, this protection was removed by preincubation with the centrifugal supernatant.

(c) If one assumes that the action of lipoxidase in the supernatant was responsible for the loss of protection, then the inhibitory action of n-propyl gallate could be expected to prevent this loss (80). NPG has been shown to be associated with an increase in the rate of formation of a substance absorbing light in the visible and ultra violet regions of the spectrum (see page 36). Cysteine, which inhibits the formation of this material (see page 38), was therefore incorporated in the blending medium used in the following type of experiment.

Two mitochondrial suspensions were prepared in the presence and absence of the inhibitors and incubated with a small volume of the respective centrifugal supernatant for 2 hours at 0° C. The rate of succinoxidase activity of each preparation was then examined in the light and dark and the results of a representative experiment appear in figure 21.

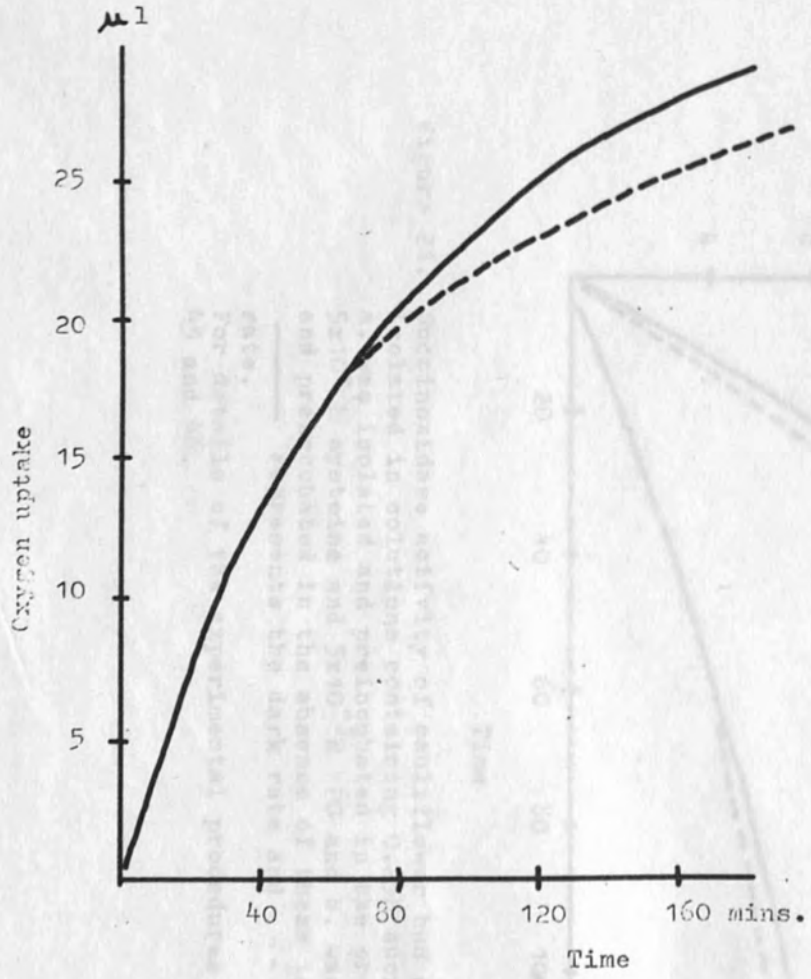


Figure 20a.

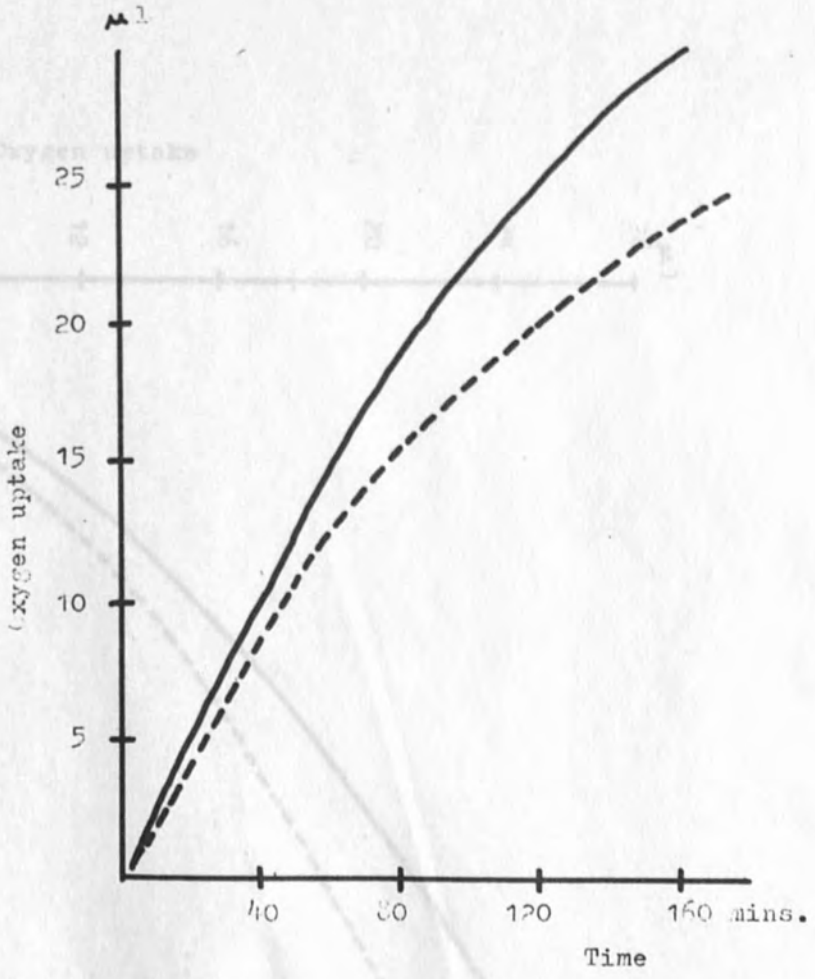


Figure 20b.

Figure 20. Succinoxidase activity of cauliflower bud mitochondrial preparation, isolated in 0.25M sucrose; a. assayed immediately following isolation of the mitochondria, b. following incubation of the mitochondria in a small volume of supernatant for 2 hours at 0°C.

——— represents the dark rate and - - - - - the light rate.

For experimental details see pages 45 and 46.

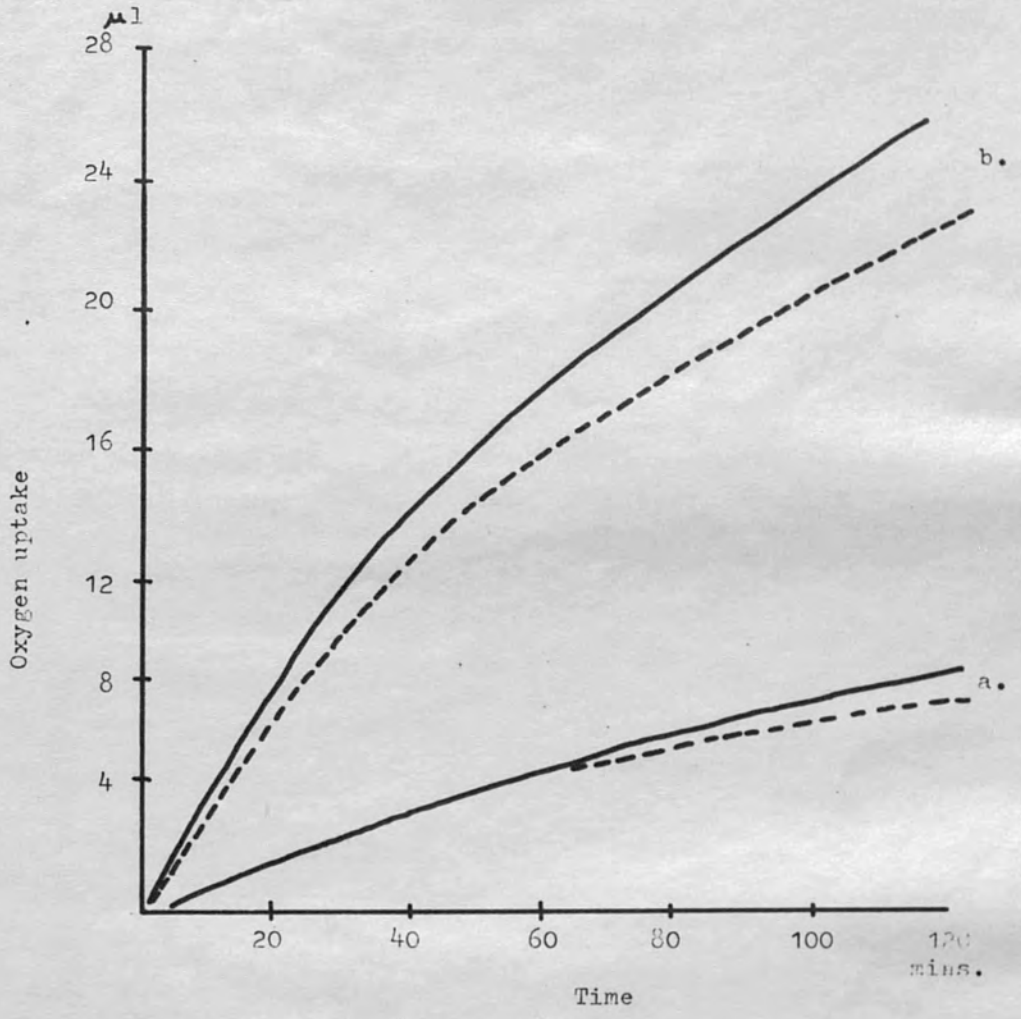


Figure 21. Succinoxidase activity of cauliflower bud mitochondria isolated in solutions containing 0.25M sucrose.
a. was isolated and preincubated in the presence of $5x10^{-3}$M cysteine and $5x10^{-3}$M IPG and b. was isolated and preincubated in the absence of these inhibitors.
——— represents the dark rate and - - - - the light rate.
For details of the experimental procedures see pages 45 and 46.

When the inhibitors were omitted from the blending medium in which the mitochondria were preincubated the rate of succinoxidase activity was immediately reduced by illumination, but protection against photosensitivity was afforded for 60 mins. to those mitochondria separated and preincubated in the presence of the inhibitors. This suggests that a lipid-oxidising enzyme, such as lipoxidase, could have been involved in the removal of protection.

(d) The above experiments have shown that light affects mitochondria that are actively respiring. The effect of preilluminating the mitochondria, in the absence of added substrate, on the subsequent rate of succinoxidase activity, measured in the light and dark, was then observed.

A washed mitochondrial suspension was divided into two similar parts. One was placed in the dark, the other illuminated for two hours, and nitrogen was slowly bubbled through both. The rate of succinoxidase activity in both suspensions was then measured in the light and the dark (see figure 22).

Preillumination in a nitrogen atmosphere resulted in an immediate reduction in the rate of respiration when mitochondria were subsequently illuminated. However, the suspension given a dark pretreatment was protected against light for 60 mins.

(e) Protection against a decrease in succinoxidase activity resulting from illumination of mitochondria resembled the protection against the lethal effects of a combination of light and molecular oxygen associated with carotenoids in some microorganisms and plant cells (see page 21). To determine whether variations in the oxygen tension during preillumination affected the photosensitivity of respiration,

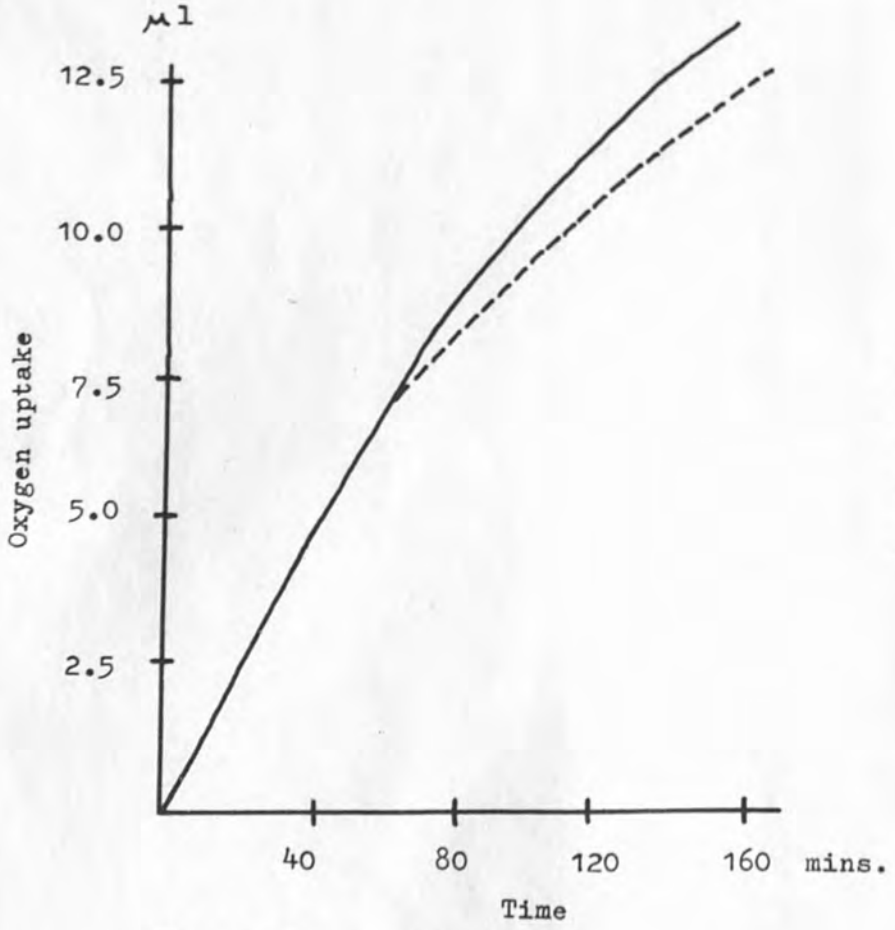


Figure 22a.

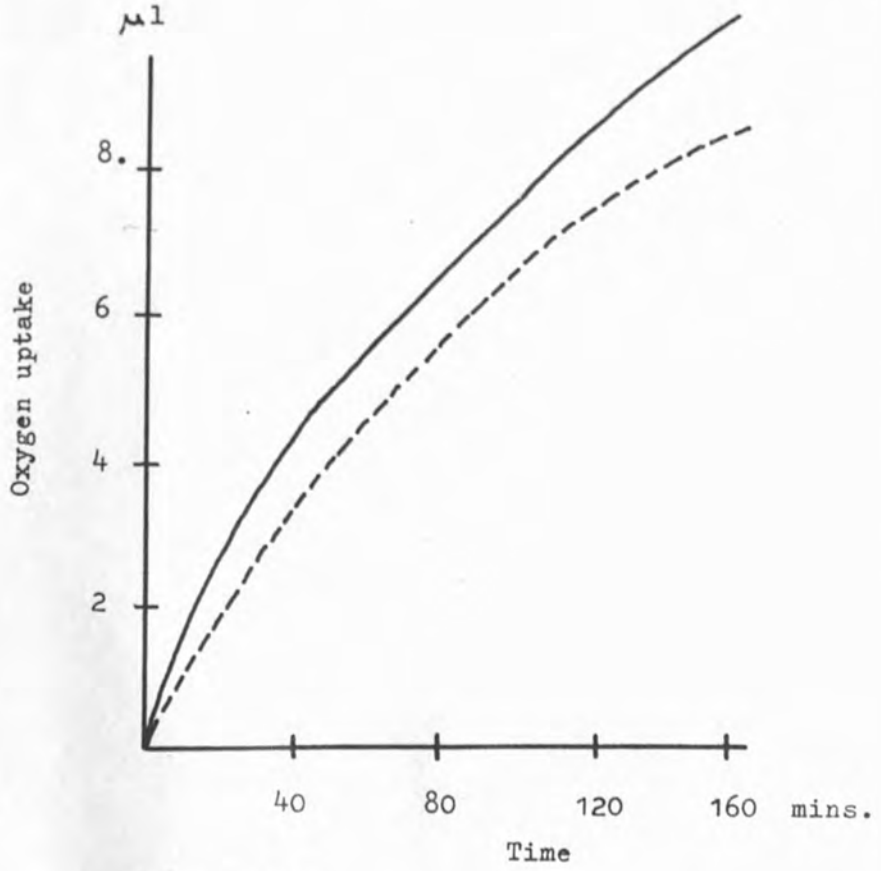


Figure 22b.

Figure 22. Succinoxidase activity of a washed mitochondrial suspension from cauliflower bud tissue, isolated in 0.25M sucrose, following incubation a. in the dark and b. in the light for 2 hours at 0°C in a nitrogen-rich atmosphere.
——— represents the dark rate and - - - - - the light rate.
For experimental details see pages 45 and 46.

the previous experiment was repeated but oxygen was bubbled through the suspensions rather than nitrogen. The results of this experiment, which appear in figure 23 resemble those obtained in the previous experiment, shown in figure 22. Thus, an increased oxygen tension during light and dark pretreatments did not affect the period of protection exhibited by respiring mitochondrial suspensions. This suggests that a protection against light in cauliflower bud mitochondria differed from that exhibited by certain plant cells and microorganisms discussed in chapter 1.

(f) Cauliflowers bought in the summer of 1967 were brown, hard, and dried because of the hot dry weather and mitochondria prepared from these possessed no detectable protection against illumination. Conversely, some Italian cauliflowers examined in the early Spring afforded protection against light for 120 mins. when assayed immediately after centrifugation, but this period was decreased to 60 mins. following preillumination or incubation with the supernatant.

(g) Many recent papers have reported the use of sucrose solutions more concentrated than 0.25M sucrose in the isolation of plant mitochondria (e.g. 98). To determine whether the results obtained in the preceding paragraphs were artifacts of mitochondria separated in dilute solutions, an early experiment was repeated on mitochondria isolated in 0.6M sucrose.

This experiment, described on page 103, in which mitochondria were assayed directly or pretreated with the centrifugal supernatant, was repeated with material prepared in 0.6M sucrose, 0.05M tris buffer (pH 7.2), and 0.001M EDTA and the results appear in figure 24. Protection remained for 120 mins. when mitochondria were assayed immediately after

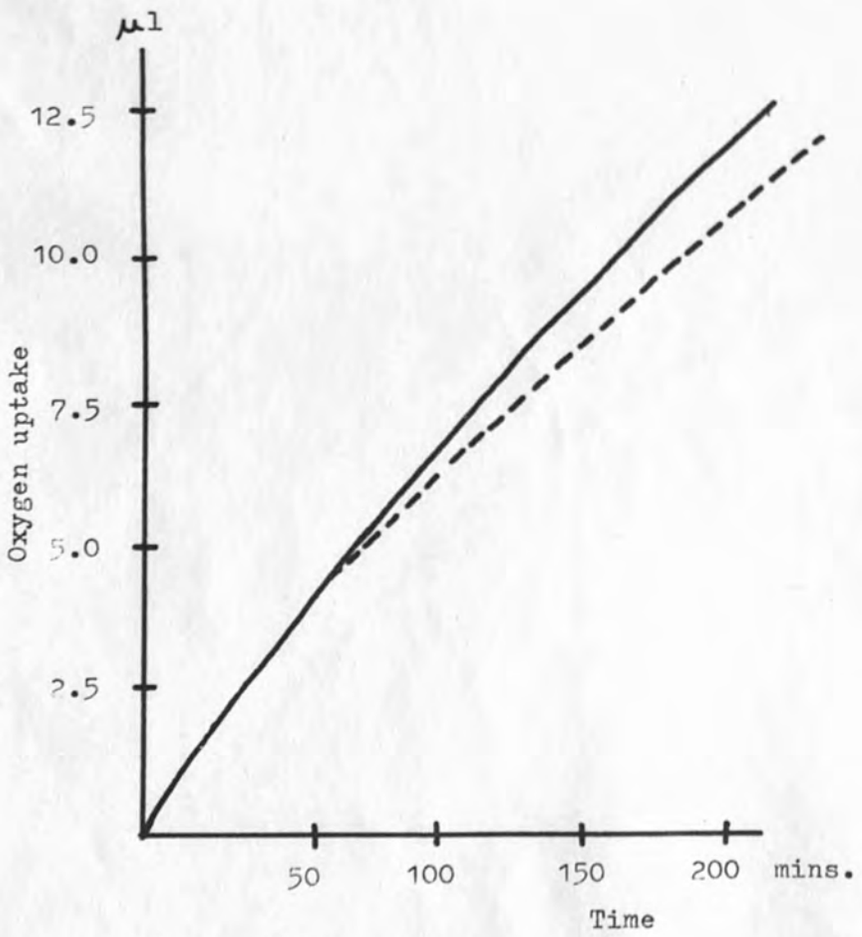


Figure 23a.

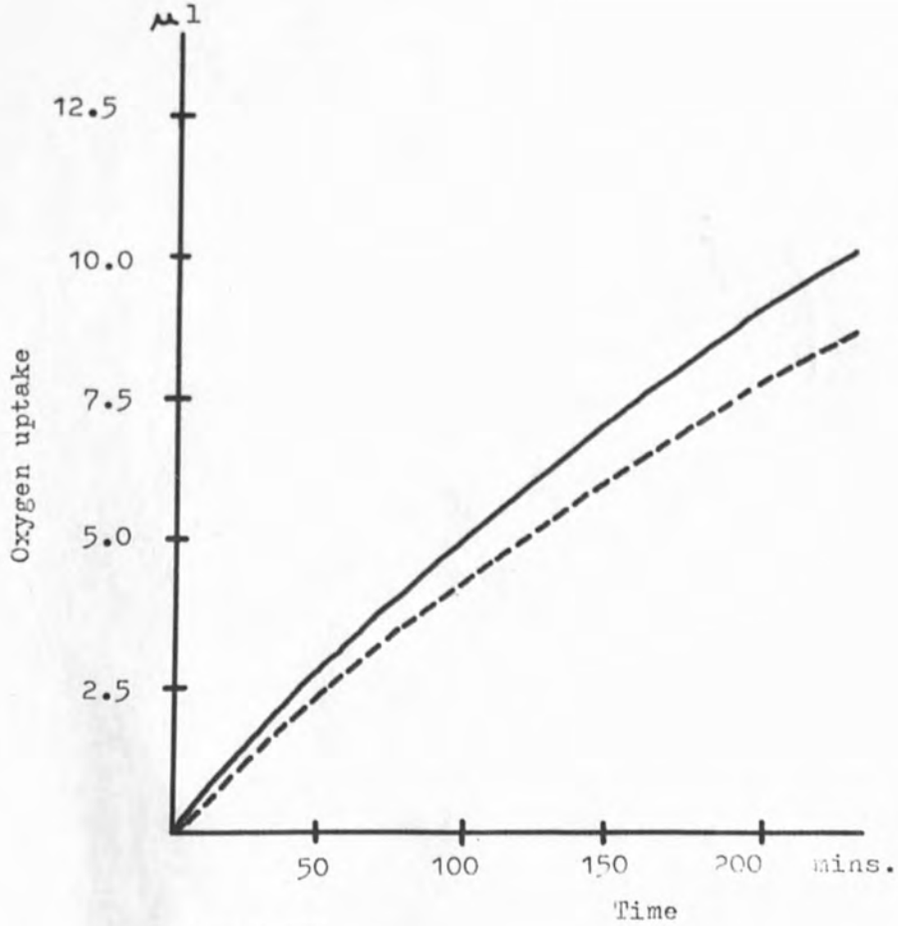


Figure 23b.

Figure 23. Succinoxidase activity of a washed mitochondrial suspension from cauliflower bud tissue, isolated in 0.25M sucrose, following incubation a. in the dark and b. in the light for 2 hours at 0°C in an oxygen-rich atmosphere.
——— represents the dark rate and - - - - - the light rate.
For experimental details see pages 45 and 46.

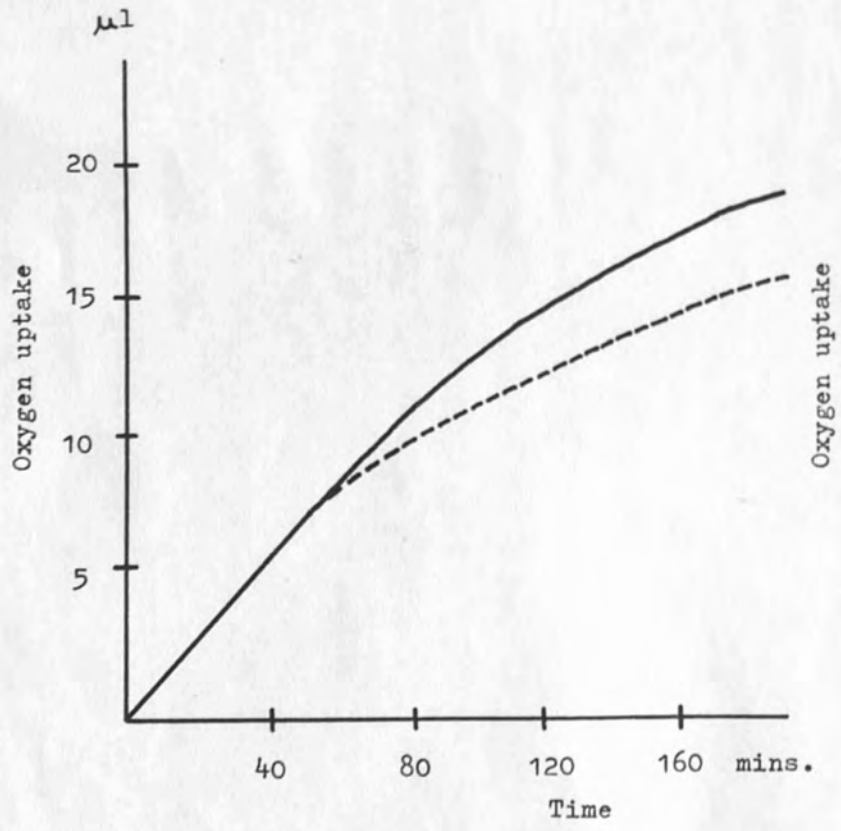


Figure 24a.

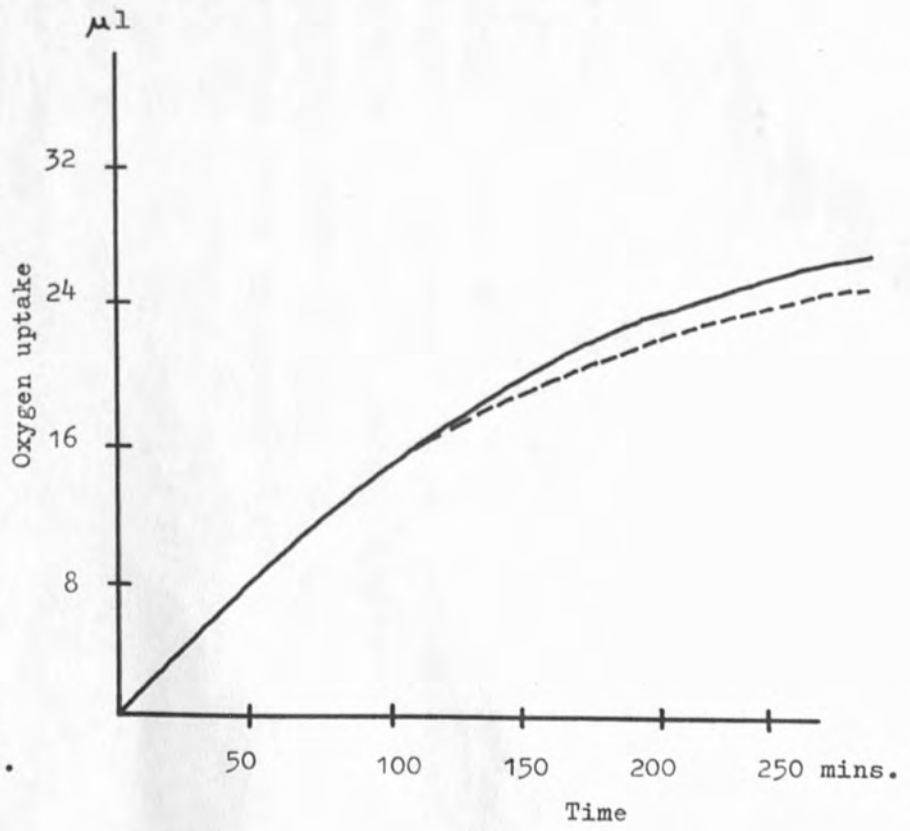


Figure 24b.

Figure 24. Succinoxidase activity of a cauliflower bud mitochondrial preparation isolated in 0.6M sucrose; a. assayed following incubation with a small volume of the centrifugal supernatant and b. assayed immediately after isolation of the mitochondria.

———— represents the dark rate and - - - - the light rate.

For experimental details see pages 45 and 46.

preparation and this period was decreased to 60 mins. by preincubation with the centrifugal supernatant.

It therefore appeared that a light-catalysed reduction in the respiration rate and protection against light for a finite period of time were not unique properties of mitochondria isolated in more dilute media.

3. Reversibility

Two experiments were performed to determine whether the inhibitory effect of illumination on respiring mitochondria was reversible. Experiments discussed previously, in which suspensions were pretreated before the assay of succinoxidase, were repeated. However, following a period of illumination during the manometric assay of succinoxidase activity, certain flasks were transferred to the dark.

In the first experiment a mitochondrial pellet was resuspended in the centrifugal supernatant and divided into four fractions. The pretreatments, given to these fractions before light and dark respiration was measured, are summarised briefly below.

(a) A fraction was incubated in the supernatant in the dark for 2 hours at 0° C.

(b) A fraction was washed to remove the supernatant, resuspended in the blending medium, and maintained in the dark at 0° C for 2 hours.

(c) A fraction was washed to remove the supernatant, resuspended in the blending medium, and illuminated for 2 hours at 0° C, being stirred by bubbles of oxygen.

(d) (c) was repeated but nitrogen bubbles were used to facilitate stirring.

Light and dark respiration were measured for 150 mins. after which exposed flasks were transferred to the dark and

subsequent succinoxidase activity was measured for a further 150 mins.. The graphs in figure 25 show the results.

Extremely good cauliflowers were used in these four experiments and therefore, as explained on page 108 , a period of illumination of 120 mins. was found to be necessary to remove protection against light when the material was assayed immediately. Preillumination or preincubation with the supernatant reduced this period of protection to 60 mins.. When the light stimulus was removed the rate of oxidation was observed to increase in all flasks, suggesting that the decrease in respiration was reversible to some extent.

A second attempt to demonstrate reversibility failed, however. In this experiment the rate of oxidation in flasks maintained in the light was compared with that in flasks which were transferred to the dark after a period of illumination of 160 mins. The results of a single set of experiments showed no increase in succinoxidase activity following the discontinuation of illumination (figure 26). However, the cauliflowers used were hard and dried and showed no initial protection against light. Therefore, the irreversibility of the decrease in respiration rate caused by light could have been a consequence of the poor condition of the cauliflowers.

4. Effect of Variations in Carotenoid Concentration on Protection against light

One can deduce from a number of the preceding experiments that carotenoids may have participated in the protection of cauliflower mitochondria against a photoreduction in the rate of respiration. Incubation with the centrifugal

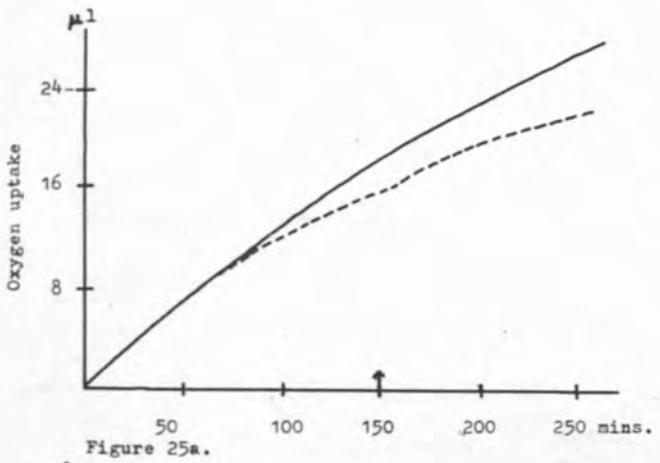


Figure 25a.

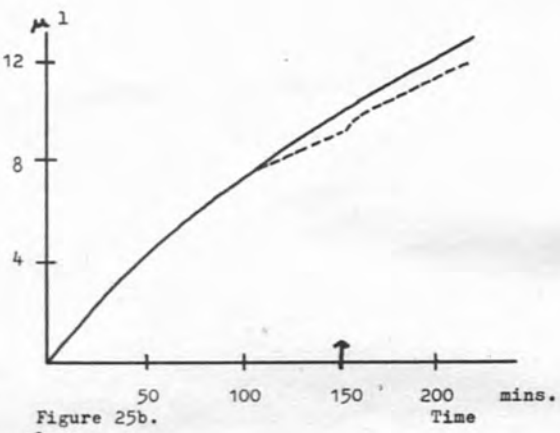


Figure 25b.

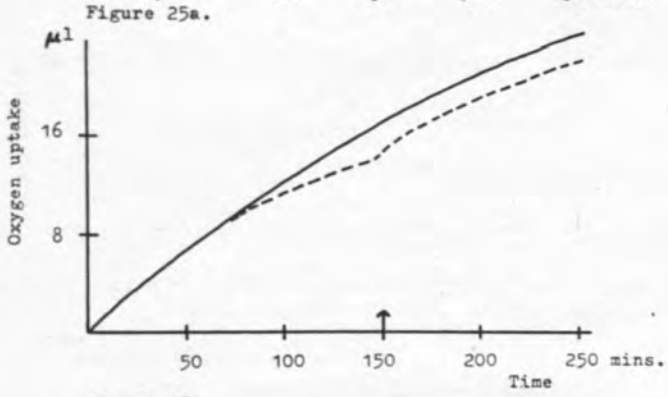


Figure 25c.

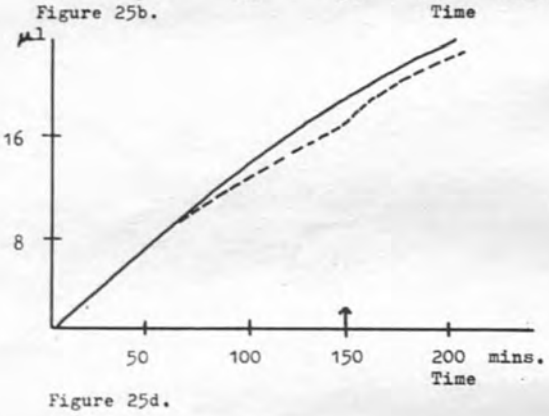


Figure 25d.

Figure 25. Reveribility of the inhibitory effect of light on respiring mitochondria.

——— represents the dark rate and ------- the light rate.

The rate of succinoxidase activity was assayed in the light for 150 mins. when the exposed flasks were transferred to the dark. Figures a,b,c,and d show the dark and light rates of succinoxidase activity of a suspension of cauliflower bud mitochondria, pretreated in the ways described below, which was added to the basic assay medium:-

a. Suspension pretreated by incubation in the centrifugal supernatant for 2 hours at 0°C, in the dark.
b. Suspension pretreated by incubation in the blending medium in the dark at 0°C for 2 hours.
c. Suspension pretreated by incubation in the blending medium in the light at 0°C for two hours, being stirred by bubbles of oxygen.
d. c. was repeated but nitrogen bubbles were used to facilitate stirring.

For experimental details, see pages 45 and 46.

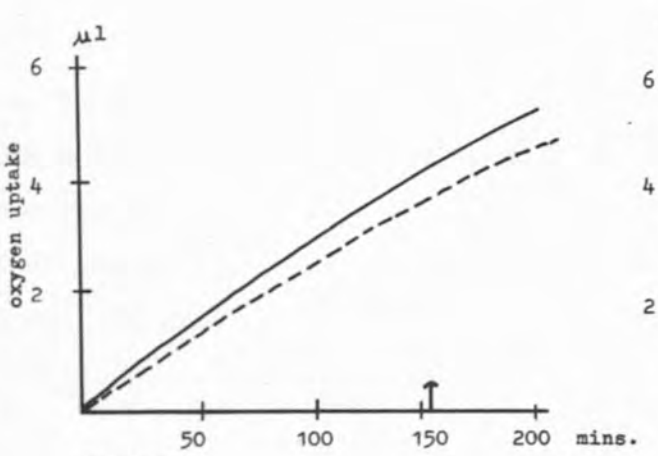


Figure 26a.

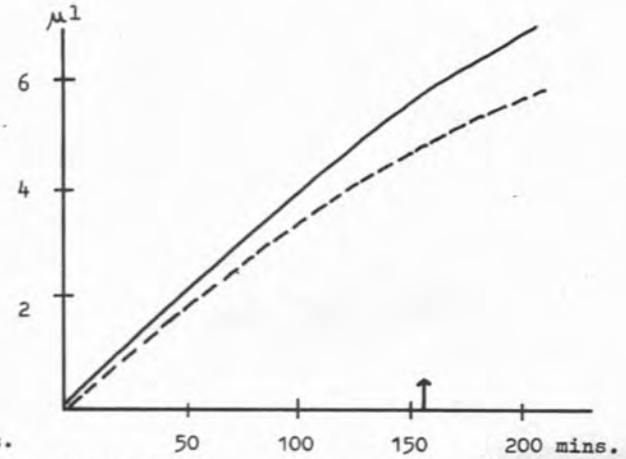


Figure 26b.

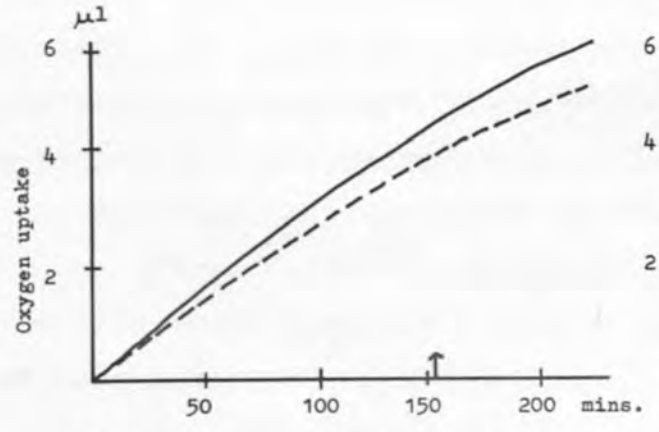


Figure 26c.

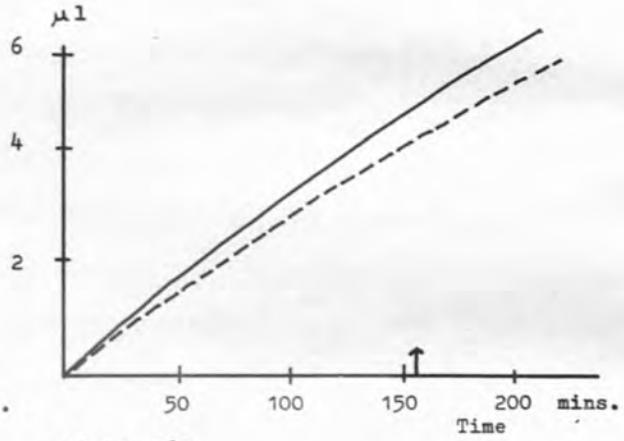


Figure 26d.

Figure 26. Reversibility of the inhibitory effect of light on the respiration of cauliflower bud mitochondria.

——— represents the dark rate and — — — — the light rate.

The rate of succinoxidase activity was assayed in the dark and light for 160 mins. when the exposed flasks were transferred to the dark. Figures a,b,c, and d show the dark and light rates of succinoxidase activity of a suspension of cauliflower bud mitochondria, pretreated in the ways described below, which was added to the basic assay medium.

a. Suspension pretreated by incubation in the centrifugal supernatant for 2 hours at 0°C in the dark.
b. Suspension pretreated by incubation in the blending medium in the dark at 0°C for 2 hours.
c. Suspension pretreated by incubation in the blending medium in the light at 0°C for two hours, being stirred by oxygen.
d. c. was repeated but nitrogen bubbles were used to facilitate stirring.

For experimental details, see pages 45 and 46.

supernatant, known to contain lipoxidase activity, removed the protection against light but when NPG was present in the centrifugal supernatant, to inhibit lipoxidase activity, this loss of protection was prevented. Experiments were therefore designed to observe the effect of variations in the carotenoid concentration on the protection of succinoxidase activity against light. A partially purified soya bean lipoxidase preparation was added to the succinoxidase assay medium in an effort to remove carotenoid but an attempt was made to protect mitochondrial unsaturated fatty acids from lipoxidase-catalysed oxidation by the addition of exogenous ammonium linoleate.

(a) A number of experiments were performed in which the ratio of the concentration of lipoxidase to linoleate was varied. In early experiments no effect was observed on the protection against light, but when the lipoxidase concentration was increased protection was lost. This loss of protection is illustrated by the results of one experiment.

Three different groups of 6 Warburg flasks were prepared containing the standard reaction medium for the assay of succinoxidase and the following additions:-

a. No addition.
b. 0.5ml. of 10^{-2}M ammonium linoleate, giving a final concentration of 2×10^{-3}M.
c. 0.5ml. of 10^{-2}M ammonium linoleate and finally 0.05ml. of the lipoxidase preparation diluted 1:5 (see page 46).

Succinoxidase activity in illuminated and darkened flasks of these three groups was examined and the results appear in figure 27.

The observed reduction in the rate of succinoxidase

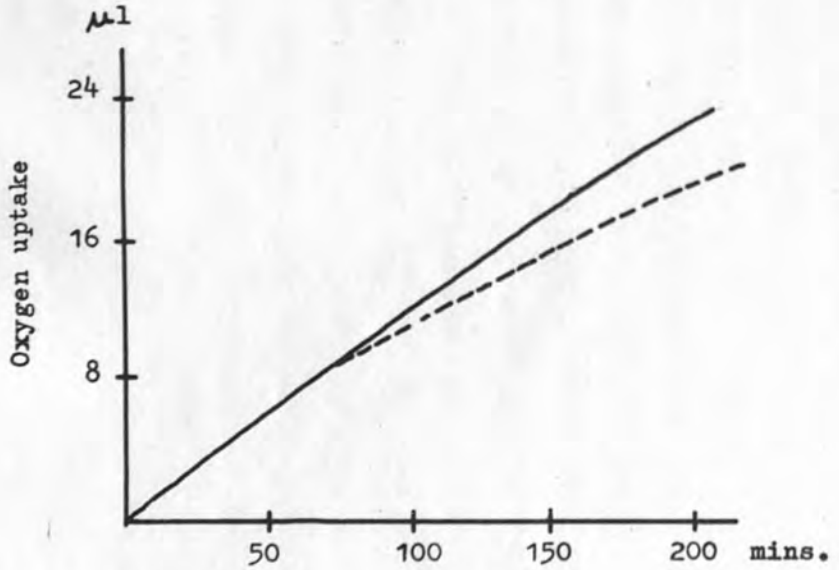


Figure 27a.

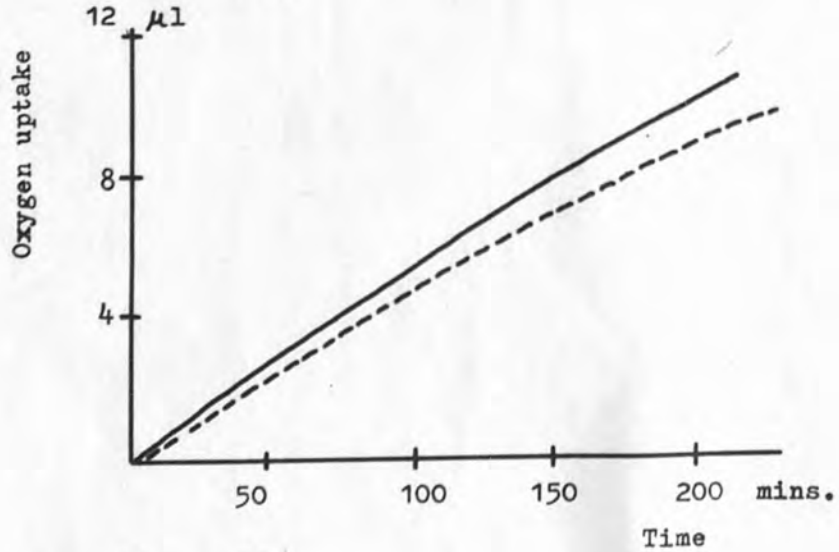


Figure 27c.

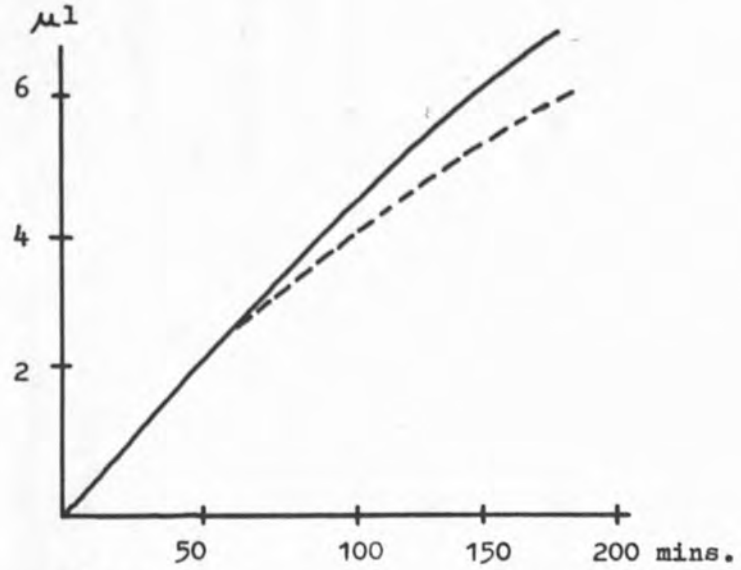


Figure 27b. Time

Figure 27. Effect of linoleate and linoleate plus lipoxidase on the length of protection of the mitochondrial suspension against light.

——— represents the dark rate and
- - - - the light rate.

Figures a,b, and c show the dark and light rates of succinoxidase activity when the following additions were made to the basic assay medium:-

a. no addition
b. 0.5ml. of 10^{-2}M ammonium linoleate
c. 0.5ml. of 10^{-2}M ammonium linoleate plus 0.05ml. of a lipoxidase preparation diluted 1:5

For experimental details see pages 45 and 46.

activity in the presence of linoleate agreed with the findings of other workers (99). However, this did not affect the period during which activity was protected against light. The combined effect of lipoxidase and linoleate removed this protection but did not alter linoleate inhibition of succinoxidase activity. The loss of protection could be attributed either to fatty acid or carotenoid breakdown.

(b) An experiment was designed to determine whether the presence of added carotenoid would safeguard mitochondria from the loss of protection against light resulting from lipoxidase activity.

The three groups of assays described in the preceding experiment were repeated simultaneously with a fourth group in which the additions to the basic assay medium were 0.5ml. 10^{-2}M ammonium linoleate, 0.05ml. of a cauliflower leaf carotenoid solution (see page 47), and 0.05ml. of the diluted lipoxidase preparation. The rates of succinoxidase activity in the dark and light were observed and gave the results shown in figure 28. Protection existed for 60 mins. in the illuminated flasks containing linoleate and lipoxidase to which carotenoid had been added but in the absence of carotenoids from these flasks protection was lost. Therefore protection against light was safeguarded from lipoxidase activity by the addition of exogenous carotenoid.

In attempts to repeat this observation, one isolated experiment produced the results in figure 29. The cauliflowers were of a very poor quality and showed no protection against light, even when assayed immediately after centrifugation (see page 108), unlike the mitochondria in the preceding experiment

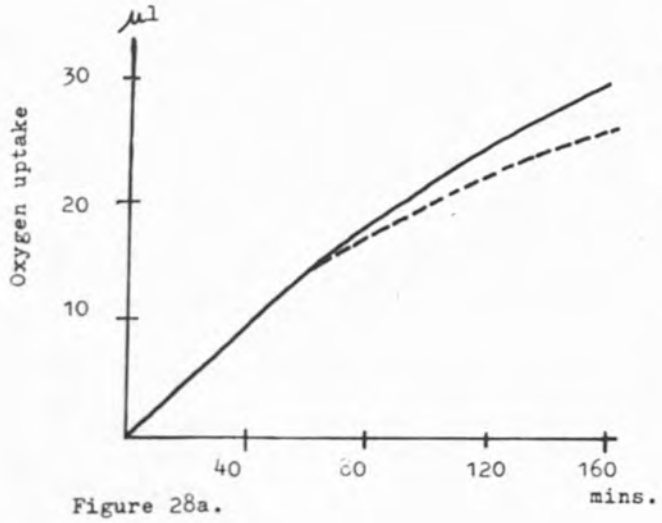


Figure 28a.

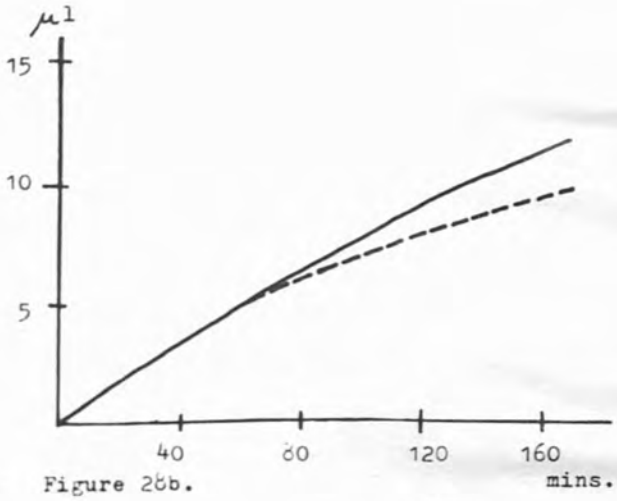


Figure 28b.

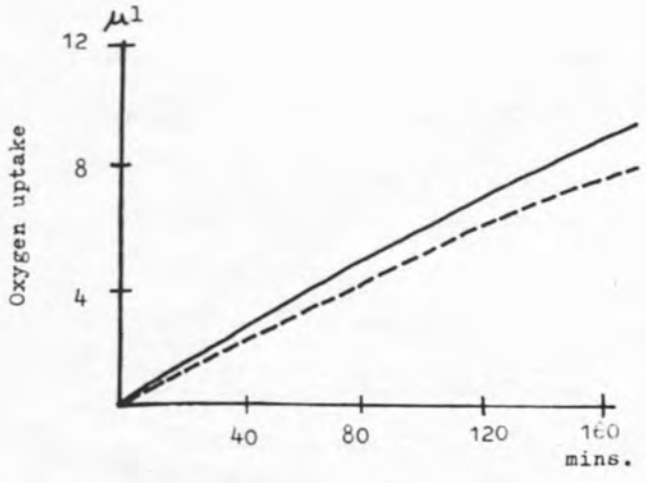


Figure 28c.

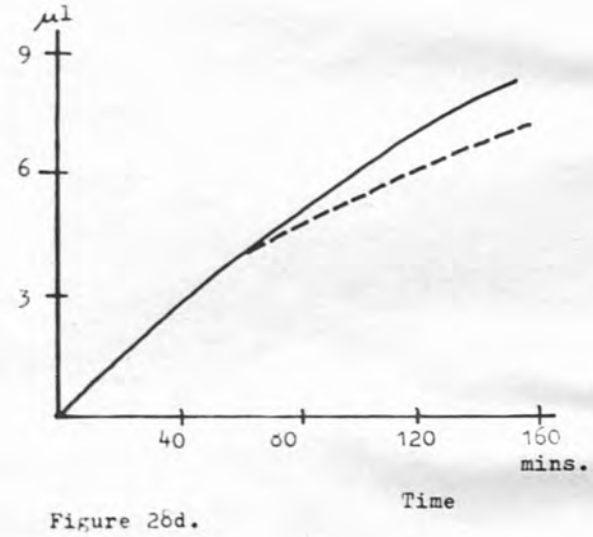


Figure 28d.

Figure 28. Safeguarding effect of a carotenoid solution against loss of protection against light resulting from lipoxidase activity.

———— represents the dark rate and - - - the light rate.

Figures a,b,c, and d show the light and dark rates of succinoxidase activity when the following additions were made to the basic assay medium:-

a. no addition
b. 0.5ml. of 10^{-2}M ammonium linoleate
c. 0.5ml. of 10^{-2}M ammonium linoleate plus 0.05ml. of the lipoxidase preparation diluted 1:5
d. The contents of c. plus 0.05ml. of a carotenoid solution

For experimental details see pages 45 to 47.

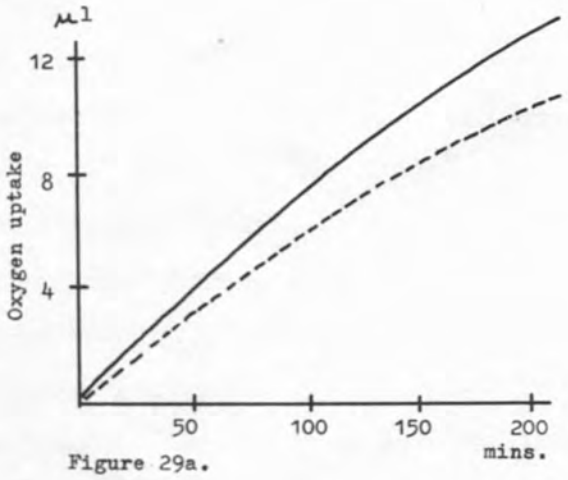


Figure 29a.

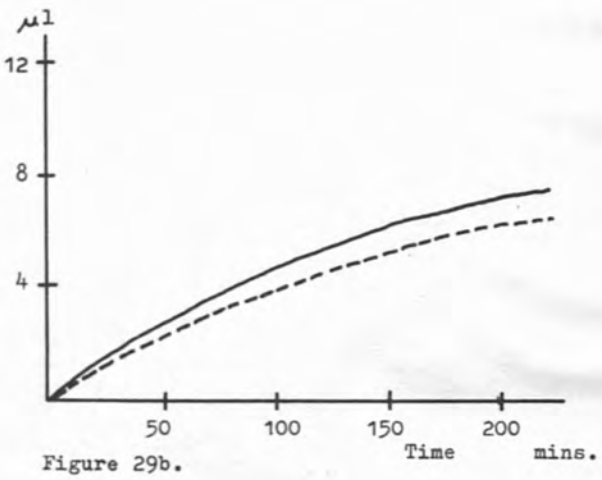


Figure 29b.

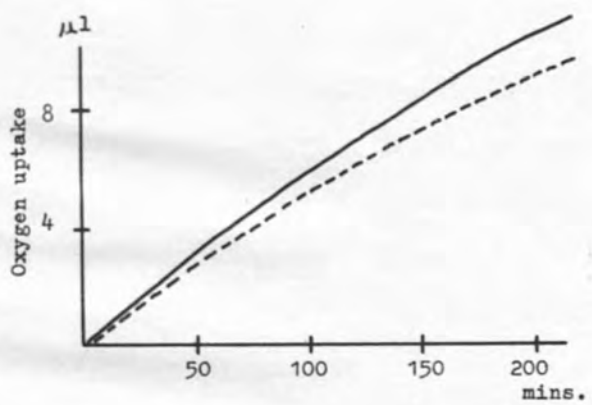


Figure 29c.

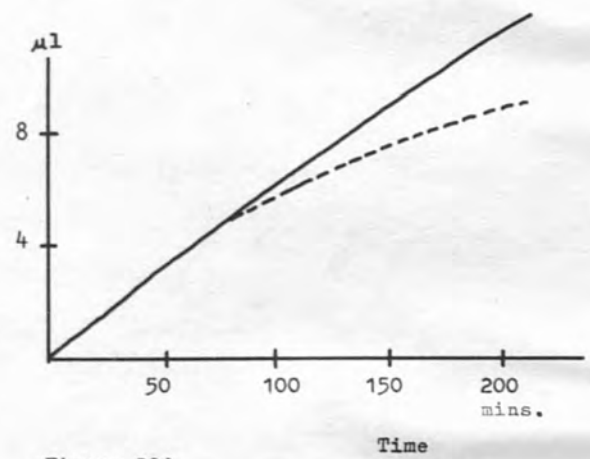


Figure 29d.

Figure 29. Safeguarding effect of carotenoid solution against loss of protection against light resulting from aging or lipoxidase activity.

———— represents the dark rate and -- ··· - the light rate.

Figures a,b,c,and d show the light and dark rates of succinoxidase activity when the following additions were made to the basic assay medium:-

a. no addition
b. 0.5ml. of 10^{-2}M ammonium linoleate
c. 0.5ml. of 10^{-2}M ammonium linoleate plus 0.05ml. of the lipoxidase preparation diluted 1:5.
d. The contents of c. plus 0.05ml. of a carotenoid solution.

For experimental details, see pages 45 to 47.

which possessed the initial protection. However, the presence of carotenoid in the medium containing linoleate and lipoxidase resulted in the development of protection which was initially absent.

(c) Maintenance of protection, resulting from the presence of the carotenoid solution in the medium containing linoleate and lipoxidase, may have been a consequence of the presence of the pigment or the solvent, an acetone/ethanol mixture (50/50, v/v.). To determine whether this was a carotenoid or solvent effect, three light and dark assays of succinoxidase activity were made in the presence of two concentrations of carotenoid and the solvent alone. All flasks contained linoleate, lipoxidase, and the basic assay medium together with the following additions, to give a volume of 2.5ml.

a. No addition.
b. 0.05ml. acetone/ethanol mixture (50/50, v/v.)
c. 0.05ml. of a concentrated solution of cauliflower leaf carotenoids in acetone/ethanol (50/50, v/v.)
d. 0.05ml. of the concentrated carotenoid solution diluted 1 in 5.

The rate of succinoxidase activity of these mitochondrial suspensions appear in figure 30. Protection was lost in the presence of linoleate and lipoxidase and was not removed by the solvent or the dilute carotenoid solution. Conversely, however, the concentrated carotenoid solution safeguarded the mitochondria against the loss of protection resulting from lipoxidase activity.

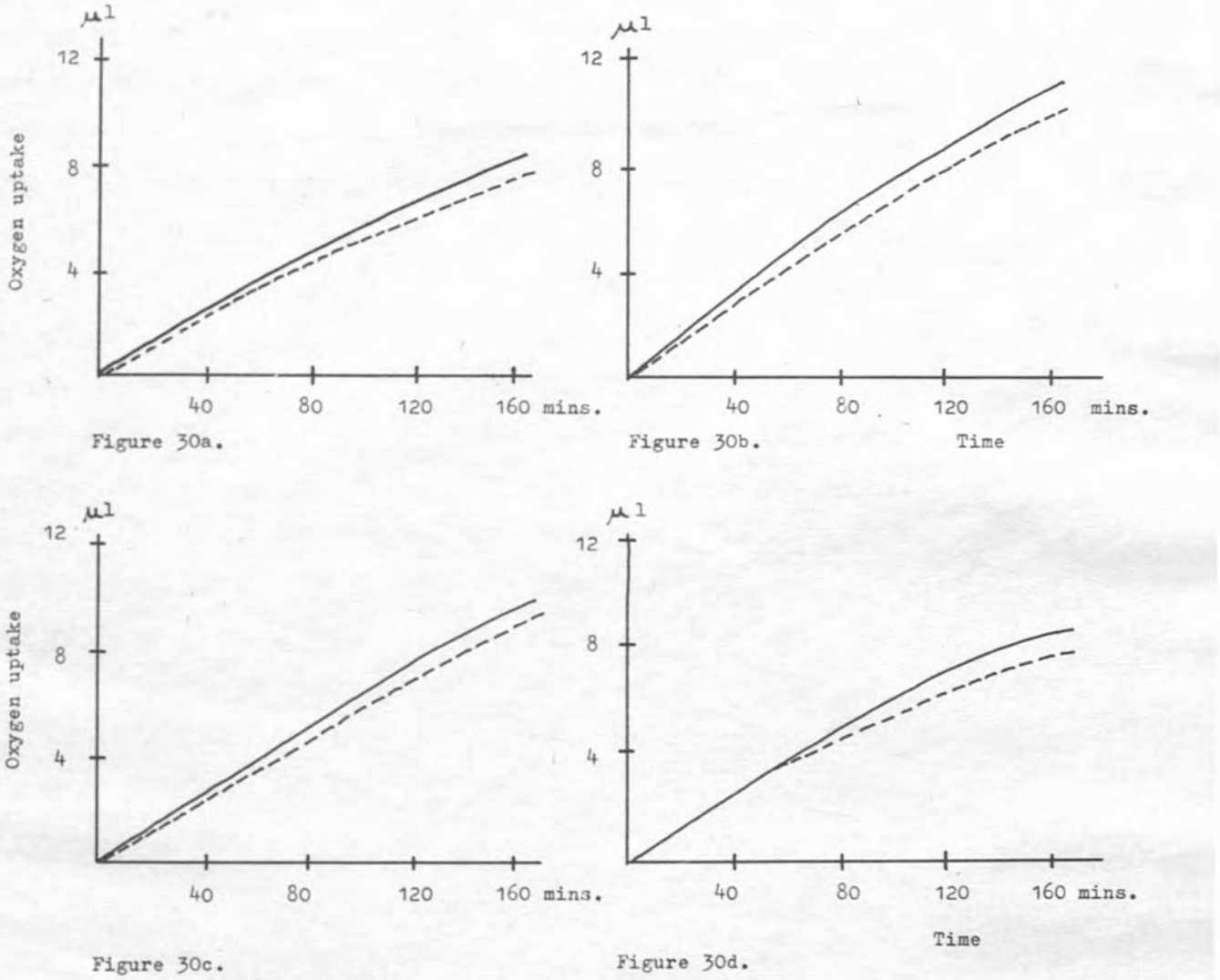


Figure 30a.

Figure 30b.

Figure 30c.

Figure 30d.

Figure 30. Differentiation between carotenoid and the solvent, acetone/ethanol (50/50, v/v), as the agent responsible for the maintainance of protection against light sensitivity resulting from lipoxidase activity.

———— represents the dark rate and - - - - the light rate.

Graphs a,b,c,and d show the dark and light rates of succinoxidase in the presence of:-
a. no addition
b. 0.05ml. acetone/ethanol (50/50, v/v) solution
c. 0.05ml. concentrated carotenoid solution in acetone/ethanol
d. 0.05ml. of the carotenoid solution diluted 1:5 with the solute.

For experimental details, see pages 45 to 47.

5. Summary

The rate of respiration of preparations of cauliflower bud mitochondria, observed by the manometric assay of succinoxidase activity, decreased in the light after varying periods. Normally, when mitochondria were assayed immediately after isolation in 0.25M sucrose, a delay of 60 mins. occurred before the appearance of the light-catalysed reduction in activity. However, the duration of this delay varied with the condition of the cauliflowers and the method by which the mitochondria were isolated.

A variety of treatments preceding the assay of succinoxidase in the dark and light affected the period of protection against light. It was reduced by incubation with the centrifugal supernatant which contained lipoxidase (this effect being blocked by NPG and cysteine in the supernatant), preillumination (which was unaffected by the oxygen tension), or by the addition of ammonium linoleate and a soya bean lipoxidase preparation. The addition of a concentrated solution of leaf carotenoids safeguarded respiration against lipoxidase-induced loss of protection and restored protection to mitochondria of old cauliflowers.

Chapter 7.

Discussion

1. Association of Carotenoids with Mitochondria

(a) Summary of Results.

A correlation has been demonstrated between the carotenoid content and succinic dehydrogenase activity of fractions of cauliflower bud homogenates separated by both differential and density gradient centrifugation. This confirmed the brief observation of Crane that carotenoids were associated with cauliflower bud mitochondria but also demonstrated that carotenoids were not associated with other subcellular fractions. A similar correlation has also been observed between the carotenoid content and succinic dehydrogenase activity of fractions separated from ox heart muscle which, following mincing, had been incubated with haemolysed blood to remove carotenoids thought to originate from the blood. This result confirmed the findings of Green (62) and Basford (63, 64) who extracted carotenoids from lipid fractions of ox heart mitochondria, but this result also excluded the possibility that the carotenoids had been derived from the blood.

Carotenoids were not detected in pig heart muscle. No correlation was found between the carotenoid content and succinoxidase activity of fractions of the yeast Rhodotorula rubra.

(b) Interpretation of results.

Three different interpretations can be forwarded to explain the results that were obtained following the homogenisation and fractionation of cauliflower buds and ox heart muscle. These are based on the assumptions that the assay of succinic dehydrogenase gave an accurate quantitative indication of the presence of mitochondria and that carotenoid

was exhaustively extracted from homogenised and unhomogenised tissue.

i. A *prima facie* interpretation of the results is that carotenoids were located in the mitochondria of these tissues *in vivo*. If carotenoids were present in mitochondria of both tissues *in vivo* and in no other fraction, then the only difference to be detected between the sedimentation patterns of carotenoid and succinic dehydrogenase activity in the centrifugal fractions would be due to experimental error. The observed standard deviations in the ratios of carotenoid content to succinic dehydrogenase activity were thought to be sufficiently small to represent no more than experimental error.

Evidence was not obtained to indicate whether carotenoids located in heart muscle cells were degraded when the minced muscle was incubated with haemolysed blood to remove blood carotenoids. However, if haemoglobin, or another blood constituent causing carotenoid oxidation, was able to penetrate the muscle fibres, it would be expected that a representative cross section of the carotenoid-containing particles would be affected. Therefore the observed sedimentation pattern of the carotenoid-containing particles would not be altered. Blood constituents were removed from the minced muscle before homogenisation and therefore carotenoids originating from heart muscle cells would not be degraded in this manner after homogenisation.

ii. A second interpretation of these results is that a second particle, which contained carotenoid, was present in the homogenates and this sedimented in the same manner as the mitochondria.

The distribution of cauliflower bud mitochondria appeared to have been affected by a number of factors including the

sharpness of the homogeniser blades and the turgidity of the cauliflowers. Therefore, if two particles with similar sedimentation properties were present then the distribution of these particles would be affected in the same way by these factors. However, this occurrence seems improbable.

A carotenoid-containing particle present in plant tissues is the plastid. Mature chloroplasts are absent from meristematic tissue, but Cunningham and Crane (93) have obtained electron micrographs of cauliflower bud tissue in which plastids were shown to be present. I have been unable to distinguish the presence of plastids in electron micrographs of centrifugal fractions, however. The density of proplastids from bean leaves was determined by Boardman and Wildman using a continuous sucrose density gradient (100). They found two bands with densities equivalent to 0.98 and 1.75M sucrose. If it is assumed that the density of these proplastids was similar to those which may have been present in cauliflower bud tissue, then the cauliflower proplastids would have been distributed at both ends of the gradient. As the majority of carotenoid-containing particles were located between 1.1 and 1.4M sucrose on the discontinuous gradient it seems unlikely that these particles were proplastids.

Heart muscle homogenates were not subjected to gradient centrifugation nor microscopic examination and therefore the presence of another particle, unlike mitochondria, which contained carotenoid was not as rigorously excluded. A lipoprotein particle containing β-carotene was isolated from ox heart muscle homogenates by Jensen and Koford (65) which appeared to "float" against the direction of the centrifugal force during separation of a mitochondrial suspension in a continuous centrifuge. Three possible sources of this lipoprotein particle are discussed by Jensen and Koford.

The first suggestion is that they may have been produced by the action of 6N KOH which had been added to neutralise acid released during homogenisation. KOH was not added to homogenates during the experiments discussed in chapter 4 and therefore particles formed by this mechanism would not be expected to contaminate the fractions analysed. Secondly, it was suggested that these lipoprotein particles originated from the action of KOH on blood and fat. When Jensen and Koford treated blood or fat with KOH in the same way as whole muscle, however, these particles were not formed. Their experiments did not exclude the possibility that these particles were formed by combination of blood carotenoids with cellular membranes. However, in the final fractionation experiment discussed in chapter 4, blood carotenoids were removed before the tissue was homogenised and therefore particles formed from blood carotenoids and cellular membranes would not be expected to have been present in the homogenate. Jensen and Koford's third suggestion was that these carotenoid-containing lipoprotein particles originated from the mitochondria.

The ox heart muscle fractions described in chapter 4 were obtained by preincubation, homogenisation in the absence of KOH, and differential centrifugation and therefore the method of preparation differed substantially from that used by Jensen and Koford. These differences may have excluded the formation of a carotenoid-containing lipoprotein particle which therefore would not have been present to contaminate the centrifugal fractions.

It seems improbable that the similarity in distribution patterns of carotenoid and succinic dehydrogenase activity in fractions of cauliflower bud and heart muscle tissue resulted from the presence of a particle, containing carotenoid, which sedimented in the same manner as the mitochondria. Heart muscle

tissue was not subjected to gradient centrifugation or electron microscopy, however, and therefore the presence of a second particle was not as rigorously excluded from this tissue as from cauliflower buds.

iii. The correlation in sedimentation patterns of carotenoid-containing particles and succinic dehydrogenase activity could have been obtained if carotenoids, released from another subcellular fraction during homogenisation, had been selectively adsorbed by cauliflower bud and ox heart muscle mitochondria.

Cauliflower bud mitochondria could conceivably have selectively adsorbed carotenoids from another subcellular source. Goodwin and Williams (66) fractionated tissue cultures of Paul's Scarlet Rose and found that in non-green tissue carotenoids were present in the centrifugal supernatant. Therefore, by comparison, carotenoids may be present in the non-particulate fraction of other plant tissues, such as cauliflower buds, and could possibly be selectively adsorbed by the mitochondria. Adsorption on the membranes in the homogenate of the tissue culture did not occur, however.

Heart muscle mitochondria may have been contaminated by carotenoids from two sources. Firstly, blood carotenoids, when they were not removed by preincubation with haemolysed blood, became randomly adsorbed on subcellular membranes during homogenisation. In experiments where carotenoids were found to be associted with mitochondria however blood carotenoids would have been almost completely degraded. Secondly, carotenoids could have been selectively adsorbed from some other subcellular fraction of the muscle cells by the mitochondria, following homogenisation.

Of the three interpretations discussed to explain the results in chapters 3 and 4 the initial one, that carotenoids are present *in vivo* in the mitochondria of both cauliflower buds and heart muscle cells, seems the most probable. Therefore, if one accepts this deduction that carotenoids are located only in the mitochondria of cauliflower buds, then, from the carotenoid analyses, one can conclude that cauliflower bud mitochondria contain the carotenoids β-carotene, lutein, violaxanthin, and neoxanthin. Similarly, ox heart muscle mitochondria probably contain α-and β-carotene and a xanthophyll.

The absence of detectable carotenoids from pig heart and the existence of a low carotenoid concentration in pig's blood suggests that these pigments may be present in heart mitochondria when they are constituents of blood lipid.

2. Association of Carotenoids with the Effect of Light on the Rate of Respiration of Cauliflower Bud Mitochondria

(a) Carotenoid Participation.

Evidence exists that carotenoids may participate in the respiration of bacteria (43), but their association with the respiration of higher organisms appears to have received no experimental support in the literature. Following observations on cauliflower buds, which suggested that carotenoids were associated with the mitochondria, the decrease in the rate of respiration caused by light was monitored and experiments were performed to determine whether carotenoids were associated with this effect. The results in chapter 6 suggest that carotenoids may have been involved in this effect of light, but they are not conclusive.

Factors which could be expected to decrease the carotenoid

content of the mitochondria also resulted in a decreased respiration rate during illumination, which suggests that the light effect may have been a consequence of a decrease in carotenoid concentration in the preparation. Incubation with the centrifugal supernatant, preillumination, the action of a lipoxidase preparation on exogenous ammonium linoleate, and aging and drying of the cauliflower buds decreased the period of protection against illumination.

It has been shown that incubation of a cauliflower bud homogenate with the centrifugal supernatant decreased the carotenoid concentration (see page 36). However, the effect on the carotenoid concentration of the other pretreatments, the condition of the cauliflowers, and also the effect of illumination during respiration were not examined. It is possible that another mitochondrial component may have been affected by the pretreatments and by illumination. For example:-

i. The centrifugal supernatant of a cauliflower bud homogenate has been shown to contain lipoxidase which decreases the carotenoid concentration, but other enzymes are also present which could have affected different mitochondrial components.

ii. The incorporation of NPG and cysteine in the blending medium not only protected carotenoids (see page 38) but also inhibited changes in polyphenols and polyunsaturated fatty acids.

iii. Preillumination could have affected the configuration or concentration of mitochondrial constituents other than carotenoids. For example, in non-photosynthetic bacteria it is thought that cytochromes absorb light which then causes oxidation of cellular constituents (57).

iv. Aging may affect carotenoids, but other changes in structure and composition are known to occur in aged mitochondria.

Support for the theory that the light effect was a consequence of a decrease in carotenoid concentration of the preparation is derived from the observations that the presence of exogenous carotenoids in the assay medium (a) safeguarded mitochondria against the loss of protection resulting from lipoxidase activity on linoleate and (b) restored protection, in the presence of lipoxidase and linoleate, which was absent from mitochondria of hard, dried cauliflowers that had exhibited no protection in the absence of lipoxidase and linoleate. The absorption spectrum of the carotenoid solution showed no contamination between 320 and 520 mμ , thus excluding the presence of oxidised coenzyme Q. However, it would have been of interest to repeat these experiments using a carotenoid solution which was chromatographically pure to exclude the possibility that a contaminant was responsible for the maintenance and restoration of protection.

The onset of sensitivity to light may have been a consequence of one of the two following causes:-

i. Illumination may have reduced the carotenoid concentration in the light-treated mitochondria below that of the dark-treated mitochondria which would have decreased the rate of respiration, assuming that carotenoids were associated with respiration. This seems unlikely as the inhibitory effect of illumination was found to be partly reversible.

ii. It may have been essential to reduce the carotenoid concentration below a certain level before the system became light sensitive. This conclusion is substantiated by the findings of the two experiments discussed immediately above where exogenous carotenoid caused the maintenance of protection against light in the presence of lipoxidase and restored protection to mitochondria from old cauliflower buds. Mitochondrial carotenoids may have

been protected by excess extramitochondrial carotenoids against the catalytic radicals formed by lipoxidase activity on linoleate and exogenous carotenoids may have become distributed in the mitochondria to replace carotenoids that had been removed during ageing.

The readdition of carotenoid could have resulted in the protection of respiration against light by an artificial method. For example, in Teale's artificial system (29) the addition of carotenoids protected chlorophyll against photo-oxidation. This only occurred when carotenoid was in sufficiently close proximity to the light-absorbing agent, however. A situation can be imagined in which carotenoids were adsorbed on the surface of the mitochondria near the light-absorbing substance which transferred excitation energy to the carotenoids resulting in protection of the cellular constituents. Artificial protection could also occur if hydroperoxides, formed by the action of lipoxidase, were mopped up by exogenous carotenoid and were therefore unable to attack the mitochondria. This could not explain the restoration of protection to aged mitochondria, however.

It therefore appears possible that carotenoids may be associated with the effect of light on mitochondrial respiration in cauliflower bud preparations, but the evidence is not conclusive.

(b) Characteristic Features of the Light Effect.

Any mechanistic theory which attempts to explain the association of carotenoids with the light effect discussed in chapter 6 must incorporate three points:-

i. Protection against a photoreduction in the rate of respiration existed for 60 mins. in mitochondria assayed immediately. This protection was lost when cauliflowers of a poor quality were used but was increased to 120 mins. when cauliflowers of a good quality were used or the mitochondria were isolated in 0.6M sucrose.

ii. In all assays, following the onset of photosensitivity, multiplication of the reduced light rate by a constant factor (which varied with each experiment), produced a rate identical to that of the control flask assayed in the dark. This is illustrated by the results of an experiment shown in table 25, in which the effect of light on respiration was observed immediately following separation of the mitochondria. The light and dark rates of oxygen uptake during the initial 60 mins. following the commencement of illumination were similar but subsequently the light rate was lower than the dark rate by a constant factor of 1.13. One can deduce, therefore, that protection against light may have been lost by mitochondria in one Warburg flask at approximately the same time and that the sensitivity of each mitochondrion did not increase with time.

iii. Results suggest that the loss of succinoxidase activity caused by illumination of actively respiring mitochondria could be reversed in the dark. Partial reversal occurred with mitochondria from good quality cauliflowers, but was not obvious when material of a poorer standard was used. The degree of reversibility of a preparation may have been inversely proportional to the length of illumination.

(c) Theoretical Concepts of Carotenoid Function in Mitochondria.

The following theoretical concepts of carotenoid function, discussed in chapter 1, may be considered as a basis for interpretation of the light effect in cauliflower bud mitochondria.

Table 25. Comparison of the dark and light rates of succinoxidase activity immediately after the beginning of illumination and after a period of 60 mins.

Time mins.	Dark O_2 uptake (μl)	Light O_2 uptake (μl)	Dark O_2 uptake (μl) after 60 mins.	Light O_2 uptake (μl) after 60 mins.	Light O_2 uptake (μl) after 60 mins. x1.13
5	0.6	0.8			
10	1.3	1.5			
15	2.0	2.1			
20	2.5	2.7			
25	3.6	3.8			
35	4.8	5.0			
45	5.8	6.0			
55	7.3	7.5			
60	7.9	8.1			
65	8.6	8.8	0.7	0.7	0.8
75	9.8	9.7	1.9	1.6	1.8
90	11.4	11.2	3.5	3.1	3.5
105	12.9	12.4	5.0	4.3	4.9
115	14.0	13.4	6.1	5.3	6.0
130	14.7	14.2	6.9	6.1	6.9
145	16.1	15.6	8.2	7.5	8.5
160	17.5	16.4	9.6	8.3	9.5
175	18.5	17.3	10.6	9.2	10.4
190	19.2	18.0	11.3	9.9	11.2

The dark and light rates of oxygen uptake are similar following the commencement of illumination. Subsequently, however, the dark rate is higher than the light rate by a constant factor of 1.3.

Mitochondria and chloroplasts are similar in many respects and perhaps the presence of carotenoids can now be added to this list of similarities. The identity of plant mitochondrial carotenoids also appear to be similar to chloroplast carotenoids, as is illustrated in table 6, which may suggest a similarity between the functions of these carotenoids. In chapter 1, a number of functions were suggested for chloroplast carotenoids which could possibly apply to plant mitochondrial carotenoids. These include protection against photo-oxidation mediated by a cellular component, electron transfer, and oxygen transfer, in which epoxy carotenoids participate.

In the absence of chlorophyll other pigments, such as cytochromes, could absorb light which may then result in oxidation of other cellular components. Transfer of the excitation energy of the cytochrome to a carotenoid molecule could protect other molecules, but could result in a simultaneous structural change in the carotenoid molecule. Following a period of illumination the carotenoid concentration could be reduced to a level which did not afford protection against light. Alternatively, carotenoids could act as a light shield, thus protecting cell components, until they were reduced to a low level where photosensitivity of respiration occurred. The light effect differed from the killing of bacteria in the presence of light and oxygen however in that it appeared to be independent of the oxygen tension.

As an alternative explanation of the results in chapter 6 the participation of carotenoids could be contemplated in electron transport or oxygen transport, the latter being supported by the fact that epoxy carotenoids were present in

the cauliflower mitochondria. Two pathways for succinate oxidation are known to be present in certain plant tissues such as potato tubers where one pathway is cyanide-sensitive and the other cyanide-insensitive (101). Two pathways may be present in cauliflower bud mitochondria, one of which is associated with carotenoid and the other independent of this pigment. Therefore, a decrease in concentration or isomerisation of the carotenoids could inactivate one pathway for electron transfer or oxygen transfer but be without effect on the functioning of the second pathway.

(d) Mechanistic theories for carotenoid participation in the light effect.

Two mechanistic theories will be discussed in which the association of carotenoids with mitochondria could explain the phenomenon observed. Firstly, the effect could be a consequence of changes in mitochondrial macrostructure during illumination. In a review by Packer and Siegenthaler (102) many physiological properties of chloroplasts and mitochondria are compared. Both swell reversibly and cardiac mitochondria become swollen ~~during illumination~~ in a manner which appears to resemble passive osmotic swelling rather than the result of metabolic activity. Swelling could be envisaged to expose carotenoids on the cauliflower bud mitochondrial surface and during the excitation of these carotenoids, resulting from illumination, they would not participate in some part of the succinoxidase system, thus reducing the efficiency of oxidation. Contraction in the dark would replace carotenoids in a favourable position for their role in oxidation. Illumination could reduce the concentration of carotenoids and therefore the degree of reversibility would be inversely proportional to the carotenoid concentration. Changes in the structure of the mitochondria,

caused by ageing and the influence of the molarity of the isolation medium, could affect the degree of swelling of the mitochondria and therefore the concentration of exposed carotenoids. This would then be expected to alter the length of time during which the succinoxidase system was unaffected by light.

Secondly, it has been suggested that two pathways for succinoxidase may exist, one independent of carotenoids and the other associated with the pigment. Two possible types of the latter pathway could be present in cauliflower bud mitochondria, one in which carotenoids were an active component and the other in which carotenoids were structurally associated with the pathway but did not participate in its activity. Regardless of which occurred, protection against light would be lost when the carotenoid concentration was decreased below a certain level. In the former of these two situations, in which carotenoids are suggested to participate, the operation of the pathway would be blocked and in the latter the degree of protection of the pathway, offered by the carotenoids, could be reduced to a point at which the pathway would become light-sensitive and inactive. Therefore, the succinoxidase activity measured following the onset of light-sensitivity would be that of the pathway which was not associated with carotenoids. When the stimulus of light was removed each system associated with carotenoids would regain activity, but the efficiency of the system would be decreased by an amount depending on the change in carotenoid concentration which would be affected by the length of illumination.

Therefore, in both theories carotenoids participate in, or are associated with, either electron or oxygen transport in a pathway for the oxidation of succinate in cauliflower bud mitochondria. In the second version of the latter theory they also protect the mitochondrial constituents against lethal photo-oxidation mediated by some other molecule.

The animal tissue examined, ox heart muscle, is never exposed to light naturally and therefore any observed protection which carotenoids may offer against photo-killing *in vitro* cannot be physiologically significant. The constituent carotenoids in this tissue were different from those of plant chloroplasts and mitochondria, which may indicate a difference in function. For example, epoxy carotenoids have been suggested to participate in oxygen transfer but in heart muscle, where the majority of carotenoids are carotenes, it is difficult to see how a similar function could occur. It would be of interest to compare the effect of light on ox heart mitochondria with the effect of light on pig heart mitochondria (which may not contain carotenoids) to determine whether carotenoids are closely related to respiration, for example in electron transfer.

3. Summary

The location of carotenoids in mitochondria is poorly documented in the literature. However, the observations discussed in this thesis extend the brief observations of Crane (67) that carotenoids are associated with the mitochondrial fraction of cauliflower bud homogenates and the observations of Green and Basford (62, 63, 64) that carotenoids are present in the mitochondria of ox heart homogenates. It is suggested that these results represent the *in vivo* association of carotenoids with mitochondria, in which case it can be stated that cauliflower bud mitochondria contain the carotenoids β-carotene, lutein, violaxanthin, and neoxanthin and ox heart mitochondria contain α-and β-carotene and a xanthophyll.

The possibilities cannot be rigorously excluded, however, that the results obtained represent the presence of a carotenoid-containing particle with the same sedimentation pattern as

mitochondria or alternatively that selective adsorption of carotenoids from another subcellular fraction by the mitochondria occurred. The presence of carotenoids in mitochondria may not be obligatory, however, as certain tissues such as pig heart muscle do not appear to contain them.

Factors which may have affected the concentration of carotenoids in cauliflower bud mitochondria affected the degree of protection of the system against a photoreduction in the rate of respiration. It would appear that carotenoids may therefore act as buffers against the lethal effects on plant mitochondria of photokilling, but association with electron transport or oxygen transport are also possible.

NOTE ADDED IN PROOF

It has been brought to my attention that in 1962 Matile* published the results of experiments in which succinoxidase activity of cauliflower bud mitochondria was inhibited by light. This light effect was reversible and the degree of inhibition appeared to decrease with time. In 1965 Klein et al** were unable to repeat these experimental results, but this may have been due to the fact that the washed mitochondria they used were in a better condition than Matile's and that the effect of illumination was observed over a short period of time.

* Matile, P. (1962). Experientia, 18, 133.

** Klein, R.M., Edsall, P.C., and Gentile, A.C. (1965). Plant Physiol., 40, 903.

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